Fig 60, 62 materials p. 1 p. 13 trans p. 14 migration p. 64 cell migration

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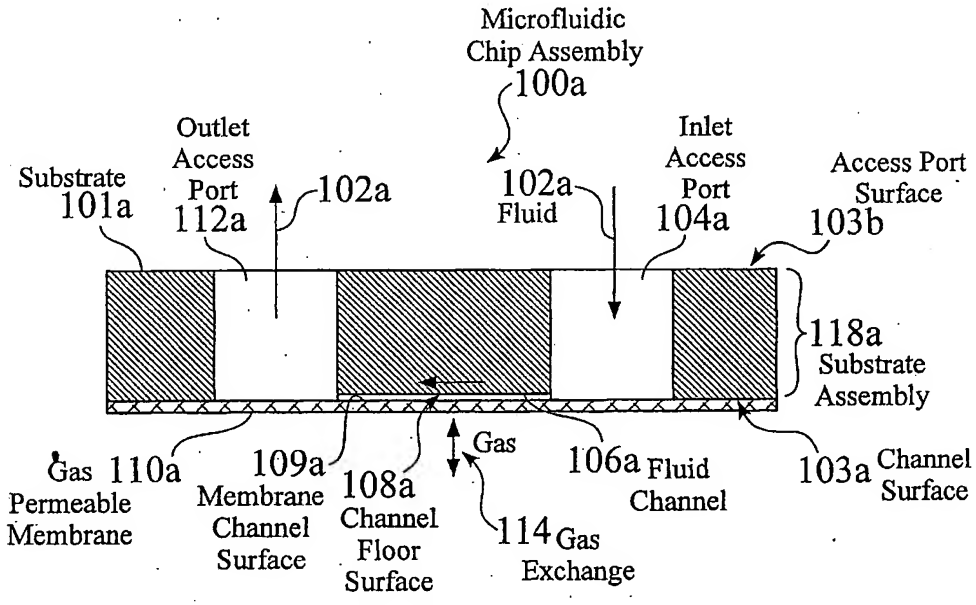

(57) Abstract: A microfluidic system for performing chemical reactions or biochemical, biological, or chemical assays utilizing a microfabricated device or "chip" is provided. The device comprises an integrated membrane fabricated from a chemically inert material whose permeability for gases, liquids, cells, and specific molecules, etc. can be selected for optimum results in a desired application.

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MICROFLUIDIC SYSTEM WITH INTEGRATED PERMEABLE MEMBRANE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to, U.S. Serial Number 60/462,957, filed April 14, 2003; U.S. Serial Number 60/434,286, filed December 16, 2002 and to U.S. Serial Number 60/453,766, filed March 10, 2003, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to microfluidic devices and systems and methods for their use. More particularly, the present invention relates to microfluidic devices and systems for performing chemical, biochemical, and cellular assays.

BACKGROUND OF THE INVENTION

[0003] Reactions and /or assays are often carried out in reaction vessels such as cuvettes, flow cells, microscope slides, or micro well plates or microplates. Macro-fluidic behavior is dominant in these types of vessels. More recently, high density micro well plates, micro arrays, and microfluidic chips have been employed when it is desired to miniaturize the reaction or assay volume. These microfluidic chips and arrays generally have been constructed from materials such as glass, plastic, or other polymers in which features controlled down to the micron level and consistent with microfluidic device operation can be readily created. One common property of these materials is that they are relatively impermeable to gases such as oxygen, nitrogen, and carbon dioxide.

[0004] Microfluidic devices have been fabricated out of poly(dimethylsiloxane) "PDMS" or silicone rubber which is highly pas permeable and can facilitate gas exchange between the interior and exterior of the chip. However, it is generally known that PDMS is highly ad/absorbent to certain hydrophobic compounds and other small molecule organic compounds such as peptides, lipids, fluorescent and non-fluorescent labels or dyes and the combinatorial and other library compounds that are often used in drug discovery assays. Adsorption and absorption of the above substances can cause undesirable levels of contamination, carry-over artifacts, depletion of compounds from solutions delivered to assay sites in biochemical and cell based assays, and background fluorescence or other signals due to absorption of fluorescent and non-fluorescent biological assays reporter groups in the PDMS. Additionally, molecules absorbed into PDMS can change their fluorescence

properties such as excitation and emission spectra, fluorescence lifetime, and fluorescence intensity, due to their interactions with the molecular structure of the PDMS interior. This can cause significant problems if it is desired to measure the fluorescence intensity or lifetime of a fluorophore within a microfluidic channel and use the information in the determination of the result of a biological or biochemical assay.

[0005] Various microfluidic chip assemblies are known in the prior art. For example, Figure 1 shows a cross section of a prior art microfluidic chip assembly 10 having a laminating adhesive layer 26. Microfluidic chip assembly 10 is fabricated from substrate material 12. Substrate 12 can be a polymer or glass. Outer layer 22 is generally a polymer but can be a thin glass layer. Adhesive layer 26 bonds substrate 12 to outer layer 22. Channel 18 is fabricated by physically removing material from adhesive layer 26 prior to assembly. Fluid 14 flows into inlet 16, through the fluid channel 18 where it passes between the substrate channel floor area 20 and surface of outer layer 22 and then exits 14 through outlet 24.

[0006] A limitation of this prior art embodiment is that it is difficult or expensive to fabricate, in practice, thin channels (< about 25 microns) and narrow channels (< about 100 microns) due to the inherent limitations of physical material removal such as physical material excision and laser cutting processes as well as the difficulties associated with alignment and lamination of structures with small feature sizes. Plastic molding and stamping techniques can be employed to fabricate adhesive layer 26 but high tooling costs and long tool fabricaton times can limit the utility of this method. Smaller feature sizes than what can be practically fabricated in the prior art example shown in figure 1 are often desirable or required in the present invention in certain embodiments. These smaller features provide the ability to control diffusion and flow rates in fluids in the channels as well as a shorter path length for diffusion of liquids or gasses in the channels or gasses in the membrane.

[0007] Another prior art chip assembly is shown in Figure 2. This microfluidic chip assembly 30 is fabricated from an impermeable support substrate material 42 thermally bonded to a hard top material 32. Fluid 34 flows into inlet 36, through the fluid channel 38 where it passes between the hard top material 32 channel floor 40 and the surface of hard support substrate 42 and then exits at 34 through outlet 44. Due to the extremely low gas permeability of the hard substrate gas exchange between the fluid and the exterior environment of the chip is negligible. Bubbles formed in the channel during priming with fluid or in operation can not readily escape other than in the initial priming process. Additionally, a dead-end channel can not be purged of gas and filled from one inlet port.

[0008] Another prior art assembly is shown in Figure 3. This microfluidic chip assembly 50 is fabricated from a hard support substrate material 60 and a soft or elastomeric material 52 into which are fabricated exemplary inlet port 504, outlet ports 67, and fluid channels 56. Fluid 54 flows into inlet 504, through the fluid channel 56 where it passes between the elastomeric material channel floor 58 and the surface of hard support substrate 60 and then exits 54 through outlet 62. Due to the high gas permeability of the elastomer and the thin channel, exchange of Gas 64 occurs readily between the fluid and the exterior environment of the chip. One of the characteristics of this embodiment of the prior art is that the relatively high gas permeability of substrate material 52 enables dead-end channels to be purged of gas and filled with fluid by application of pressure to a fluid-filled an inlet port connected to the dead-end channel.

[0009] Figure 4 is a top view of another prior art microfluidic chip assembly 70 having a concentration gradient generator 80 connected to a microfluidic channel 82. As taught by an embodiment of the prior art, a microfluidic chip assembly 70 is fabricated from a hard support substrate material 72 and a soft or elastomeric material (PDMS) 74. Microfluidic chip assembly 70 is fabricated from a hard support substrate material or coverslip 72 and a soft or elastomeric material 74 into which are fabricated inlet ports 76, outlet port 84, and fluid channel 82.

[0010] Reagents 75 flow from inlets 76, through the "gradient generator" 80 and into fluid channel 82 and then exit through outlet 84. Between the time the fluids enter at gradient generator inlets 76 or cell inlet 78, the fluid passes between the elastomeric material channel wall 98 and the surface of coverslip 72 as seen in figure 5. However, small molecules such as those commonly used as test reagents in drug screening assays are readily and rapidly adsorbed to the surface and absorbed into the volume of the PDMS material from which the channels are fabricated. This effect is dramatically exacerbated by the high surface to volume ratio in the microfluidic channels of the gradient generator 80 and channel 82. The net effect is that test compounds are absorbed into the PDMS in an unpredictable way. This is highly undesirable for screening assays both since test compound may not be predictably delivered to its destination and there may be undesirable carry-over if the fluid is switched from one test compound to another.

[0011] Another problem with art as taught by the prior art taught is that the large size of the gradient generator makes the device impractical to "scale-up" to provide large numbers of assays as is routinely required for drug screening assays, i.e., preferably to hundreds or even many thousands of assays per day. Moreover, the prior art does not teach a method for

doing a screening assay with a test compound but only a method for inducing chemotaxis in a gradient of chemoattractant formed in a channel with neurtophils attached therein. Last, the device taught by the prior art provides only a one dimensional chemoattractant concentration gradients to be formed in the channel thus limiting the amount of information available to be obtained.

[0012] Figure 5 is a partial cut-away perspective view 88 of the microfluidic chip assembly of figure 4 demonstrating neutrophil 96 chemotaxis in a microfluidic channel 82. As taught be an embodiment of the prior art, gradient of chemoattractant is created in fluid channel 82 by gradient generator 80 using the "split and combine" method known by those skilled in the art. Neutrophils 96 disposed in channel 82 and attached to coverslip 72 exhibit chemotaxis in response to the concentration gradient transverse to the direction of the flow and migrate in the direction of increasing concentration of the chemoattractant.

[0013] As described above, integrated valves have been implemented using hard structures made from silicon or silicon dioxide and soft materials like PDMS. Valves made from hard materials (i.e., elastic modulus > 1E11 Pa) must be large to obtain the deflection needed to open and close with practical actuators and to control realistic solution volumes. Unfortunately, the use of hard materials leads to sensitivity to leakage due to trapping of particulate matter. Valves made with soft materials like PDMS (i.e., elastic modulus < 1E6 Pa) structures are easy to actuate, small in size, and are relatively insensitive to leakage due to trapping of particulate. However, these materials, particularly PDMS have a high affinity for ab/adsorption of solvents and other small molecules as described previously above and since PDMS is highly gas permeable, bubbles can form in microfluidic channels that are in close proximity to the valve. Finally PDMS has extremely high permeability to water vapor, particularly when one side of the PDMS is in contact with liquid water. This high water permeability leads to rapid evaporation from microfluidic channels which must somehow be managed in order for microfluidic devices made from DMS to be successfully used in applications which require extended residence times of water in the channels.

[0014] To facilitate low-cost and high-quality chemotaxis assays, there is a need for microfluidic devices or systems that are inert to materials contained therein particularly library test compounds, DMSO, tracers and other common reagents used in biological assays, that resist bubble formation, that reduce or compensate for evaporation of water from the channels within the chip, that minimize the amount of test compounds, reagents, cells and chemoattractant required, provide for increased cell respiration and cell viability, and that evenly distribute test compound and other common reagents while providing for generation

of a range of chemoattractant concentrations and gradients (to accommodate for normal biological operating range) and means to compensate for variations in flow rate from any cause that can affect the generated chemoattractant concentrations and gradients so as to insure that accurate measurements of chemotaxis can be made in screening applications where many separate measurements are made, for example up (96, 384, 1536, and 3456 measurements per microplate) and each measurement is compared to a set of positive negative and positive controls. Therefore, there is a need for microfluidic devices or systems that provide for increased cell respiration and cell viability, that are inert to materials contained therein, that resist bubble formation during valve actuation and channel priming, and that reduce or control the relative rate of evaporation of water from the channels within the chip and that provide the capability to perform chemical, biochemical, and cellular assays in the presence of reagent concentration gradients.

SUMMARY OF THE INVENTION

[0015] The present invention provides a microfluidic system for performing chemical reactions or chemical, biochemical, biological, or cellular assays utilizing a microfabricated device or "chip" and methods for generation of concentrations and concentration gradients of assay reagents while compensating for variations in flow rates, concentrations and concentration gradients due to intrinsic and extrinsic factors such as described in the background section above. The present invention also includes a method for pre-loading, storing, and/or freezing cells and other reagents in chips which can be stored either at the manufacturer or at the end user until needed for uses including but not limited to assays, reference standards, archival samples, sensors, monitors, diagnostics, and instrumentation systems. The present invention facilitates low-cost and high-quality cell migration assays, is inert to materials contained therein particularly library test compounds, DMSO, tracers and other common reagents used in biological assays, resists bubble formation, reduces and compensates for evaporation of water from the channels within the chip, minimizes the amount of test compounds, reagents, cells and expensive chemoattractant or other biomolecules required, provides for increased cell respiration and cell viability, and evenly distributes test compound and other common reagents while providing for generation of a range of chemoattractant or other assay reagent concentrations and gradients (to accommodate for normal biological operating range) and means to compensate for variations in flow rate from any cause that can affect the generated chemoattractant or other assay reagent concentrations and gradients so as to insure that accurate measurements of cell migration or other biochemical or cellular assay readouts can be made in screening

applications where many separate measurements are made, for example up (96, 384, 1536, and 3456 measurements per microplate) and each measurement is compared to a set of positive negative and positive controls.

[0016] In one embodiment, cells are flowed into a microfluidic channel either under externally applied pressure or by the pressure generated by pipetting a column of fluid into an input well. Once the cells have entered the channel and flowed to the cell perfusion or assay region, flow is stopped either by switching off the external pressure with a valve, neutralizing the external or internal pressure with an opposing pressure, or by equalizing the applied pressure to all relevant fluidic nodes of the assay device. In the simplest case a fluid column equal in height to input well is pipetted into an output well causing flow to stop or at least slow down to the point where the cells will settle on the chip surface. Depending on the orientation of the microfabricated device, cells will either settle on the membrane or the substrate side. After the cells have settled, and attached, and stabilized, the assay begins. In this embodiment, one or more tracer is added to the flow stream, each in adjacent laminar flow regions. The tracer particles can be detected optically, mechanically, thermally, or electrically. Examples of tracer molecules are small molecular weight dyes, labeled peptides, labeled carbohydrates, labeled beads etc. For example, visible particles could be chosen as could fluorescent dye molecules. Since the rate of diffusion of a tracer molecule into an adjacent flow stream is governed by both its diffusion coefficient and the flow velocity, the flow velocity can be determined using an optical measurement of the tracer diffusion into an adjacent flow stream or by the ratio of the inter-diffusion of multiple tracers in adjacent flow streams. Once the flow rate is known, the concentration and concentration gradient can be calculated. In addition, the concentration and concentration gradient can be determined from a measurement of the level of tracer present across the channel. By measuring concentration and concentration gradient in each channel and designing the system to provide a range of concentrations and concentration gradients, the position in the channel with the proper concentration and concentration gradient can be retrospectively determined and the position along the channel with the desired concentration and concentration gradient can be selected for comparison to assay points in other channels.

[0017] In other embodiments, the tracer could be identified by fluorescence intensity or fluorescence lifetime. Tracers could be non fluorescent absorbing dyes, optically visible reflective, refractive, or absorptive particles, quantum dots, chemical indicator molecules which measure surrogate gradients designed to mimic the chemoattractant gradient. For example a surrogate gradient could be formed using a dissolved gas and the gradient could be

sensed with a gas sensing indicator molecules, the primary requirement is that the diffusion coefficient of the tracer system is in the same range as that of the chemoattractant. Another important requirement is that interference from the material in the channel does not degrade the quality of the measurement of the concentration gradient. For example, library test compounds are sometimes fluorescent as are certain assay reporters. The tracer used to normalize the chemoattractant concentration and gradient should be selected to be resistant to the type of interference expected to be encountered. Tracers with long fluorescence lifetimes or long wavelengths can sometimes be less susceptible to the effects of compound fluorescence; however, the effects of static and dynamic quenching can be problematic for long lived fluorescent tracers. Lastly, since the dimensions of microfluidic channels are so small, there are generally not many molecules present at physiological concentrations. This would hold true for both the interferers and the tracers. The type and amount of tracer used should be optimized for each situation.

[0018] In other embodiments, the tracer could be free molecules in solution or it could be chemically attached to the chemoattractant in such a way that it does not interfere with its ability to bind to its target chemotactic receptor site. By monitoring the tracer on the chemoattractant, it should be possible to determine the concentration and gradient at any point along the channel. Additionally, by attaching larger size molecules to the chemoattractant without compromising its bioactivity, it would be possible to lower its diffusion coefficient and thereby allow desired concentration gradients to be generated at lower flow rates.

[0019] To assist in the determination of the exact spatial position of the concentration and gradients of the chemoattractant, the present invention includes a series of calibrated reference (or fiducial) marks along the channels which can be used as a reference by the imaging optical system. Use of such marks will allow the determination of the location of a desired concentration and concentration gradient despite the face that the position of the desired concentration and concentration gradient may vary from one assay site to the next.

[0020] In another embodiment, cells are flowed into the chip, the flow is stopped, the cells attach, are allowed to grow, the media may then be changed, and the cells are frozen. Freezing protocol must be optimized for the best result and generally involves freezing and thawing processes that are optimized for a given chip and cell type. In an alternate embodiment, the cells may not be frozen but held at a lower temperature that would cause the cells to enter into a state of stasis. Chips containing frozen cells or cells in stasis can be suitably packaged so as to be archived at the factory or transported to their final users and

where they can be stored and eventually thawed and used for assays without the need to load cells into the chip. Frozen chips can be used for sample archival and ultimately for screening, life science research, personalized medicine including therapy optimization, genotyping, and gene expression, and other medical diagnostics applications.

[0021] The accompanying drawings, which are incorporated in and form a part of this specification, illustrate embodiments of the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a cross section of a microfluidic chip assembly having a laminating adhesive layer.

Figure 2 shows a chip cross section of a microfluidic chip assembly having a impermeable support substrate and top cap.

Figure 3 shows a cross section of a microfluidic chip assembly having a hard support substrate and microfabricated elastomeric body.

Figure 4 is a top view of a microfluidic chip assembly having a concentration gradient generator connected to a microfluidic channel.

Figure 5 is a partial cut-away perspective view of the microfluidic chip assembly of Figure 4 demonstrating neutrophil 96 chemotaxis in a microfluidic channel.

Figure 6 shows a cross section of a microfluidic chip assembly according to the present invention.

Figure 7 is a cross section of an exemplary microfluidic chip assembly comprising an exemplary dead-end channel.

Figure 8 is a cross section of a microfluidic chip assembly with an exemplary gas manifold

Figure 9 is a cross section of a microfluidic chip assembly with a mechanical valve actuator in the open position.

Figure 10 is a cross section of a microfluidic chip assembly with a mechanical valve actuator in the closed position.

Figure 11 is a top view of microfluidic chip assembly showing the location of a valve along a channel.

Figure 12 is a top view of a fixture comprising an array of mechanical valve actuation posts and means for mechanical alignment to a chip.

Figure 13 is a side view of the fixture of figure 12.

Figure 14 is a top view of an alternate embodiment for actuating a plurality of valves according to the present invention.

Figure 15 is a side view of the valve structure shown in figure 14, wherein the valve actuation ports are disposed in a layer opposite to the fluid access ports.

Figure 16 is a side view of the valve structure shown in figure 14, wherein the valve actuation ports are disposed in the same layer as the fluid access ports.

Figure 17 is an exemplary process flow chart for the fabrication of a chip according to the present invention.

Figure 18 is a cross sectional view of a starting glass substrate.

Figure 19 shows the application of a layer of masking material to the substrate.

Figure 20 shows the application of photoresist to the masking material.

Figure 21 shows a process step to expose and develop the photoresist leaving areas of exposed masking material.

Figure 22 shows a process step to etch the exposed masking material to form a patterned etch mask.

Figure 23 shows a process step to etch the glass exposed by the patterned etch mask.

Figure 24 shows a process step to strip the patterned etch mask.

Figure 25 shows the application of a sand blast mask.

Figure 26 shows a process step to fabricate holes by sand blasting areas exposed by a sand blast mask.

Figure 27 shows a process step to remove a sand blast mask.

Figure 28 is a cross sectional view of a starting membrane.

Figure 29 shows a process step to apply a bonding layer to a membrane.

Figure 30 shows a process step to bond a membrane and bonding layer to a substrate.

Figure 31 shows an alternative process step in which bonding monolayers are applied to a substrate and a membrane.

Figure 32 shows a process step to bond a substrate to a membrane, through applied bonding monolayers.

Figure 33 shows a perspective view of an exemplary industry standard 96 well micro titer plate.

Figure 34 shows a cross sectional perspective view of a microfluidic well assembly comprising an assembled chip mounted in a 96 well microplate compatible well frame.

Figure 35 shows a partial cut away perspective view of the microfluidic well assembly of figure 34.

Figure 36 shows a partial cut away top view of a microplate chip package having conical wells.

Figure 37 shows a partial cross section of the microplate chip package of figure 36.

Figure 38 is a cross section of a chip to be laminated.

Figure 39 is a cross section of a well frame to be laminated.

Figure 40 shows a process step to apply an adhesive to the well frame of figure 39.

Figure 41 shows a process step to laminate the chip of figure 39 to the well frame of figure 40.

Figure 42 is a perspective view of a re-usable well frame assembly for sealably mounting and operating a chip according to the present invention.

Figure 43 shows an architectural block diagram for a system to operate a microfluidic chip according to the present invention.

Figure 44 is an architectural block diagram for a system to operate a microfluidic chip according to the present invention in a robotically automated laboratory environment.

Figure 45 is a plan view of a microscope slide sized substrate having an array of access ports in standardized locations on the substrate.

Figure 46 illustrates a cut-away plan view of exemplary 2 and 4 port standard unit cells having standardized access port locations as shown in figure 45.

Figure 47 is a plan view of an exemplary standard unit cell placed to optimally utilize the standard array of access ports shown in figure 45.

Figure 48 is an expanded view of a 3-1 combiner standard unit cell layout utilizing the standardized access port locations shown in figure 45.

Figure 49 shows an exemplary array of 96 standard unit cells with each unit cell having up to 4 access ports disposed in a standard 384 well format.

Figure 50 illustrates exemplary 4 and 8 port standard unit cells each with alternative channel network configurations, any of which being suitable for placement into a standard microplate format such the one shown in figure 49.

Figure 51 illustrates a unit cell array in a standard 96 well format, the array comprising 24 repetitions of 4 port standard cells, each unit cell comprising a 3-1 combiner structure.

Figure 52 illustrates a unit cell array in a standard 384 well format, the array comprising 96 repetitions of 4 port standard cells, each unit cell comprising a 3-1 combiner structure.

Figure 53 illustrates a unit cell array in a standard 1536 well format, the array comprising 96 repetitions of 4 port standard cells, each unit cell comprising a 3-1 combiner structure.

Figure 54 illustrates exemplary routing networks distributing two common reagents to each site in the exemplary array of 96 standard unit cells described in figure 49.

Figure 55 is a partial view of an exemplary network of channels for routing two common reagents to exemplary standard unit cells with 3-1 combiner structures suitable for substitution into the standard unit cell array of figure 54.

Figure 56 illustrates a ring routing network for distribution of a common reagent within an 8 port standard unit cell.

Figure 57 shows a linear channel network within an 8 port standard unit cell.

Figure 58 illustrates an 8 port standard unit cell in a microplate format with a ring channel network.

Figure 59 shows a 4 port standard unit cell in a microplate format with a ring channel network.

Figure 60 illustrates a 4 port unit cell in a microplate format with an H channel network.

Figure 61 shows a star channel network for distribution of a common reagent within a multiple port standard unit cell.

Figure 62 illustrates a linear channel network within a multiple port standard unit cell equivalent to the star channel network of figure 61.

Figure 63 shows a multiple port standard unit cell in a microplate format with a star channel network.

Figure 64 illustrates an exemplary serial channel network for distributing a common reagent to a plurality of unit cells within an 8 port standard unit cell with isolation valves in the open position.

Figure 65 shows the standard unit cell of figure 64 with the isolation valves in the closed position and the common reagent distributed to and trapped within the assay region of each unit cell in the plurality of unit cells.

Figure 66 illustrates an exemplary serial channel network for distributing a common reagent to multiple 2-1 channel unit cells within an 8 port standard unit cell with isolation valves in the open position.

Figure 67 shows the standard unit cell of figure 66 with the isolation valves in the closed position and the common reagent distributed to and trapped within the assay region of each 2-1 channel unit cell.

Figure 68 illustrates an exemplary embodiment of three H equivalent structures with an integrated parallel network for distributing a common reagent to the assay region of three unit cells within an 8 port standard unit cell.

Figure 69 shows an exemplary 3-1 structure wherein each of the three channels carries a common reagent and merges into a single main channel.

Figure 70 shows the 3-1 structure of figure 69, wherein each of three channels carries a common first reagent, in which a second reagent is added to the outer channels causing a standing concentration gradient to form in the main channel.

Figure 71 illustrates the concentration of a first and second reagent in the structure of figure 69 at an upstream location in the main channel relative to the merge point.

Figure 72 illustrates the concentration of a first and second reagent in the structure of figure 69 at a downstream location in the main channel relative to the merge point.

Figure 73 shows a 3-1 combiner structure similar to that of figure 69 wherein each of the three channels is carrying a common reagent and cells have been loaded into an assay region in the main channel.

Figure 74 shows the 3-1 combiner structure of figure 73 wherein a second reagent has been added to the outer channels causing the cells to migrate in response to the concentration gradient of the second reagent formed along the main channel.

Figure 75 illustrates an exemplary method for loading cells into the main channel from the center channel of the structure similar to figure 69.

Figure 76 shows the 3-1 combiner structure of figure 75 wherein a second reagent has been added to the outer channels.

Figure 77 illustrates an exemplary method for loading cells into the main channel of an H structure from one of the side branch channels.

Figure 78 shows the 3-1 combiner structure of figure 79 wherein a second reagent has been added to one of the branch channels of the structure of figure 79.

Figure 79 shows a plan view of an exemplary chamber shaped to efficiently purge an assay region in a microfluidic perfusion chamber.

Figure 80 illustrates an exemplary dead-end channel along a main channel running between two access ports which is inefficiently purged by the flow in the main channel.

Figure 81 shows an exemplary valve covering the center region of an exemplary H structure.

Figure 82 shows an exemplary method of loading cells into an H structure from a side branch, the H structure having a valve in a central region to trap cells.

Figure 83 shows the structure of figure 84 with the valve closed and the cells trapped in the two dead-end channels created by the closed valve.

Figure 84 shows the structure of figure 85 after flow has been allowed to continue and wash away the cells not trapped in the dead-end channels.

Figure 85 shows the structure of figure 86 after performing an assay, wherein a second reagent is added.

Figure 86 shows an embodiment of a two compartment device wherein cells are loaded into a first compartment through a first channel.

Figure 87 shows the two compartment device of figure 88 after the introduction of a reagent that induces cell migration from the first compartment into the second compartment.

Figure 88 provides an illustrative example of bell shaped and saturating dose-response curves.

Figure 89 shows overlapping standing gradients of a first and a second reagent in the main channel of a 3-1 structure wherein the first and second reagents are fed from the left and right channels, respectively.

Figure 90 shows an exemplary method wherein multiple cell types are loaded into the main channel of a 3-1 combiner structure.

DETAILED DESCRIPTION OF THE INVENTION

I. The Device

A. Overview

[0022] Figure 6 is a cross sectional view of a microfluidic chip assembly 100a according to the present invention. Substrate assembly 118a, i.e. a fabricated substrate is fabricated from substrate material 101. Fabricated substrate 118a comprises an inlet access port 104a and an outlet access port 112a extending between a channel surface 103a and an access port surface 103b. A fluid channel 106a is located on channel surface 103a of substrate 101a, extending between inlet access port 104a and outlet access port 112a defining a channel floor surface 108a. A gas permeable membrane 110a is sealably attached to channel surface 103a of fabricated substrate 118a defining a membrane surface 108a within fluid channel 106a. Fluid 102a flows into inlet 104a, through fluid channel 106a where it passes between the channel floor 108a and membrane surface 109a and then exits through outlet 112a. Due to the relatively high gas permeability of the membrane and thin channel depth, exchange of gas 114 occurs between the fluid and the exterior environment of the chip. Bubbles formed in the channel during priming with fluid or in operation escape through the membrane.

B. Substrate

[0023] Suitable substrate materials are generally selected based upon their compatibility with the conditions present in the particular operation to be performed by the device. Such conditions can include a range of or extremes of pH, temperature, ionic concentration, solvent tolerance and application of electric fields. Additionally, substrate materials are also selected for their inertness to critical components of an analysis to be carried out by the system. Useful substrate materials include, e.g., glass, quartz, ceramics,

and silicon, as well as polymeric substances, e.g., plastics. Although quartz or glass is preferably used as the substrate material 101, in other embodiments silicon or another inert material of similar physical qualities can be used. For certain applications where features with high aspect ratios are desired or possibly for low-cost applications, plastic is the preferred substrate although materials with crystal structure such silicon can be anisotropically etched to provide high aspect ration features..

[0024] In the case of polymeric substrates, the substrate materials may be rigid, semi-rigid, or non-rigid, opaque, semi-opaque, or transparent, depending upon the use for which they are intended. For example, systems which include an optical or visual detection element are generally fabricated, at least in part, from optically transparent materials to allow, or at least, facilitate that detection. Alternatively, optically transparent windows of glass or quartz, e.g., may be incorporated into the device for these types of detection. Optically transparent means that the material allows light of wavelengths ranging from 180 to 1500 nm, usually from 220 to 800 nm, more usually from 250 to 800 nm, to have low transmission losses. Such light transmissive polymeric materials will be characterized by low crystallinity and include polycarbonate, polyethylene terepthalate, polystyrene, polymethylpentene, fluorocarbon copolymers, polyacrylates (including polymethacrylates, and more particularly polymethylmethacrylate (PMMA), and the like. Additionally, the polymeric materials may have linear or branched backbones, and may be crosslinked or non-crosslinked. Examples of particularly preferred polymeric materials include, e.g., polydimethylsiloxanes (PDMS), polyurethane, polyvinylchloride (PVC), polystyrene, polysulfone, polycarbonate, polymethylmethacrylate (PMMA) and the like. Fluorosilicones and other fluoropolymers, and fluoropolymer coated polymer materials are also potentially desirable substrate materials due to the ability to fabricate high aspect ration structures that resist ad/absorption. High aspect ratio structures are those with channel width/depth >.3. High aspect ratio structures can be fabricated by embossing, molding, casting, or soft lithography with polymeric materials.

[0025] In a preferred embodiment, the materials used to fabricate the microfluidic devices are selected for resistance to ad/absorption by aggressive organic solvents, certain acids and bases, biomolecules such as nucleotides, peptides, proteins, lipids, natural product screening libraries etc. as well as and small molecule combinatorial compound libraries which have a tendency to absorb into conventional materials. Resistance to ad/absorption of organic solvents and combinatorial library compounds minimizes undesirable levels of contamination, carry-over artifacts, depletion of compounds from solutions delivered to assay

sites in biochemical and cell based assays, and background fluorescence due to absorption of fluorescent and non-fluorescent biological assays reporter groups in the substrate material. Resistance to organic solvents, acids, and basis enables the use of microfluidics with combinatorial, synthetic organic and other chemistries. The ability to select materials with desired surface characteristics may also be important in certain chromatography applications.

[0026] In a preferred embodiment, substrate comprises an optically clear material with low background fluorescence and birefringence, allowing optical interrogation of the chamber.

C. Surface Modifications

[0027] It may be desirable to modify the surface of the device to reduce or enhance the various driving forces (e.g., electroosmotic, electrokinetic, electrophoretic, and the like) through the channel and the like, to reduce or enhance analyte adsorption. It may be desirable to modify the surface of the device to reduce or enhance the ability or rate which cells attach to channels or chambers within the device. The channel floor surface 108a can be functionalized; the membrane channel surface 109a can be functionalized; or the surfaces of both can be functionalized. In the latter case, the surface of the membrane channel can be modified in the same manner or in a different manner from the functionalization of the channel floor surface.

[0028] The use of different surface modifications may serve to increase the sensitivity of the device to particular species of interest. For example, the device can be readily modified by reducing or enhancing analyte adsorption to the walls of a channel, chamber, access port, well, or reservoir to allow for the probing of many different molecular interactions. Methods of silane surface chemistry developed in the past twenty years can be applied to the substrate or the conduit, allowing hundreds of different molecules to be grafted onto the device's surface. The surface can be modified with a coating by using thin-film technology based, for example, on physical vapor deposition, thermal processing, or plasma-enhanced chemical vapor deposition. Alternatively, plasma exposure can be used to directly activate or alter the surface and create a coating. For instance, plasma etch procedures can be used to oxidize a polymeric surface (i.e., a polystyrene or polyethylene to expose polar functionalities such as hydroxyls, carboxylic acids, aldehydes, or other reactive moieties).

[0029] The coating may comprise an organic thin film. Methods for the formation of organic thin films include in situ growth from the surface, deposition by physisorption, spin-coating, chemisorption, self-assembly and plasma-initiated polymerization from gas phase. For example, a material such as dextran can serve as a suitable organic thin film. Other

thinfilms include lipid bilayers; monolayers of polyarginine or polylysine, fibronectin, collagens of various types, surface adhesion molecules such as integrins; self-assembled monolayers; and the like. The coating may cover the whole surface of the device or only parts of it, e.g., including channels, conduits, chambers, access ports, wells, reservoirs, etc. A variety of techniques for generating patterns of coatings on the surface of a support are well known in the art and include, without limitation, microfluidics printing, microstamping, and microcontact printing.

[0030] Additional references describing methods for surface modification include US Patent No. 4,680,201; U.S. Patent No. 5,433,898; U.S. Patent No. 6,056,860; EP 665,430, EP 452,055; and Encyclopedia of Polymer Science and Engineering "Adhesion and Bonding", Vol. 1, pp. 476 et seq (Wiley Interscience, 1985), each of which is incorporated herein by reference.

D. Membrane

[0031] The microfluidic chip assembly comprises a gas permeable, chemically inert membrane 110a that overlays the substrate assembly 118a to sealably enclose the various channels, chambers, and the like. The integrated permeable membrane 110a confers the ability to control the transport of liquids or gases into and out of the solutions contained within a microfluidic environment. A reasonable design criterion for selecting membrane physical properties is the estimated time required to fill a one cm dead-end channel. For embodiments including certain chemical, biochemical, and cellular assays and assuming a channel fill time of <5 minutes, the membrane is preferably be selected from the group including but not limited to relatively chemically inert materials with an oxygen permeability greater than 10 Barrer units and water permeability less than 5 times the oxygen permeability. Exemplary materials which meet these criteria include polyolefins such as poly methyl pentene and amorphous fluorinated polymers such as Teflon AF or CYTOP. Elastomeric materials such as silicones are preferred in applications where the low elastic modulus of silicone is desired and its tendencies toward molecular ad and absorption can be accommodated.

[0032] The CGS unit of measurement for gas permeability in general use for membranes and other thin films is the Barrer. Permeability is defined to be the gas flow rate multiplied by the thickness of the material divided by the area and by the pressure difference across the material. The Barrer is the permeability represented by a flow rate of 10E-10 cubic centimeters per second times 1 centimeter of thickness, per square centimeter of area and centimeter of mercury difference in pressure (volume at standard temperature and pressure, 0

°C and 1 atmosphere), 1 Barrer = 10-10 cm2·s-1·cmHg-1, or, in SI units, 7.5005 x 10-18 m2·s-1·Pa-1.

[0033] Table 1 below shows relevant permeabilities for candidate membrane materials.

Membrane materials	Nitrogen	Oxygen	Carbon Dioxide	Water	Water /Oxygen
LDPE (Low Density Polyethylene)	0.969	2.88	12.6	90	31
HDPE (High Density Polyethylene)	0.143	0.4	0.36	12	30
Poly(methylpentene)	7.83	32	92.6	60	1.9
PP (Polypropylene)	0.44	2.3	9.2	51	22
Silicon rubber 10%filler	227	489	3240	43000	8.8
Polystyrene	0.8	2.63	10.5	1200	461
Teflon AF 2400	490	990	2800	4026	4

[0034] Membranes used in embodiments containing low dead-volume valves and relatively small physical size having the ability to control and mix reagents in a microfluidic environment are preferably comprised of chemically inert materials with elastic modulus of between 1E6 Pa and 1E9 Pa and are fabricated with a thickness in the range of 10 to 100 microns and more preferably in the range of 10-50 microns.

[0035] For example, the Teflon fluoropolymer series from DuPont Chemical (e.g., PTFE, FEP Teflon AF, etc.) are good choices for the integrated membranes since they are highly resistant to the chemical compounds listed above and are readily bondable to the substrate as are or other halogenated polymer materials or polymer coatings from other sources and can also can be selected if they can provide the desired level of gas permeability. Teflon can be bonded or laminated to substrates such as glass or Kapton by a combination of heat treatments and the use of adhesives or silanizing agents. Numerous Teflon applications notes available from DuPont Corporation. For example, DuPont provides recipes and specifications for laminating Teflon FEP to various substrates including glass and Kapton (polyimide) and is shown below for reference.

[0036] Teflon AF has desirable properties for use as a gas permeable membrane in embodiments of the present invention since it exhibits low mechanical creep, displays high optical clarity, and has very gas permeability (comparable to silicone rubber). DuPont also provides guidance for processing Teflon AF which is included for reference. DuPont reports

that Teflon AF can be bonded to glass substrates with a fluorinated silane (ref) Teflon® AF is easier to process than other fluoropolymers. It is mechanically stiff over a broad temperature range and has a low *cold flow*. Because Teflon® AF has limited solubility in perfluorocarbon solvents, it can be cast into thin-film, pinhole-free coatings, with no sintering, and only low heat needed to drive off residual solvent. It also can be applied using spin, spray, brush, or dipping techniques. Teflon® AF can be molded at relatively low temperatures by extrusion, pressing, or injection molding, in typical fluoropolymer molding equipment. In addition, it can be dissolved in selected perfluorinated solvents for the production of highly uniform thin films and coatings through spin coating and other techniques. Another property of Teflon AF is that its index of refraction is around 1.3 which is lower than that of water at 1.33. A microfluidic channel, coated on all sides with Teflon AF would function as a waveguide with an NA of about .28. This embodiment could be used to collect light generated within the waveguide by a fluorescent label or reporter group as part of an assay or sensor and direct it toward a photodetector located at the end of the waveguide. For example, fluorescent indicators for substances such as carbon dioxide, oxygen, pH, calcium, etc. are readily available from companies like Molecular Probes, Inc. 29851 Willow Creek Road, Eugene, OR 97402.

[0037] An alternative perfluorinated fluoropolymer with similar properties to Teflon AF is CYTOP, which is available from: Bellex International Corp., Wilmington, DE. CYTOP has an optical transmittance of >95% from 200 nm; a refractive index of 1.34 (D-line); and a dielectric constant of 2.1.

[0038] Alternatively, the membrane may be formed from polydimethylsiloxane (i.e., silicone rubber) or even gas permeable contact lens material. Many formulations of silicone rubber are commercially available, some having properties optimized for compatibility with certain process chemicals, biocompatibility, and the like. Other formulations are available for injection molding and yet still other formulations are available with fluorinated structural elements conferring high resistance to specific process chemicals or biomolecules. One of the big differences between silicone rubber and materials like Teflon AF is that the water permeability of silicone is 60 times higher for water than for oxygen whereas with Teflon AF, the water permeability is only 4 to 5 times higher than for oxygen. The higher water permeability of silicone can make a significant difference in certain applications.

[0039] In certain applications the effective permeability can be reduced by either backing the membrane with a second impermeable material or by reducing the concentration gradient of the diffusion species by bringing a substance in contact with the back side of the

membrane that drives diffusion in the opposite direction so as to cancel out the permeability of the membrane. For example, if it was desired to allow atmospheric gases to diffuse in and out of the chip, but evaporation of water was to be minimized, the exterior surface of the membrane would be kept in contact with a layer of water of sufficient thickness to allow gas diffusion while sourcing the water molecules required to prevent evaporation from the inside of the chip.

[0040] Additionally, the membrane surfaces can be derivatized to allow coupling molecules such long chain hydrocarbons or antibodies for chromatography or fibronectin for growing cells as discussed below.

II. Other Assembly Embodiments

A. Assembly with Dead-end Channel

[0041] Figure 7 is a cross section of an exemplary microfluidic chip assembly 120 comprising an exemplary dead-end channel 106b. In an alternate embodiment of the present invention, microfluidic chip assembly 120 is fabricated with a dead end channel 106b and inlet port 104b. Substrate assembly 118b is fabricated from substrate material 101b. Fluid 126 flows into inlet port 104b, through fluid channel 106b where it passes between channel floor 108b and surface 109b of gas permeable membrane 110b until fluid fills the entire channel 106b. Due to the relatively high gas permeability of the membrane and the thin channel depth, gas exchange 130 between the external environment and the fluid in the channel is relatively rapid. Additionally, gas 134 trapped in the channel exits through the area of the membrane covering the channel and not filled with fluid.

B. Assembly with Gas Manifold

[0042] Figure 8 is a cross section of a microfluidic chip assembly 210 including an exemplary gas manifold 222. Assembly 210 is an embodiment of the present invention designed to bring a gas or a mixture of gasses 224 into diffusive communication with the contents of a microfluidic channel 106c and hence in contact with the contents 16 of channel 106c. Assembly 210 is comprised of optional gas manifold 222 affixed to membrane 110c. Fluid flows into inlet 104c and then through channel 106c where it passes between the surface of substrate 101c and gas permeable membrane 110c where it encounters the contents of the channel 16 and then exits from the outlet 112c.

[0043] During operation of the device, a gas mixture 224 enters the manifold input 226, passes over the membrane 110c and then exits from the manifold through output 228. Due to the relatively high gas permeability of the membrane and the thin channel depth, exchange of oxygen and carbon dioxide is rapid compared to what is required to insure that

the gas is in diffusive communication with the contents of the channel.

[0044] The gas is delivered to the contents of the channel in order to carry out assays to study the effects of various gasses on the contents of the channel. In alternate embodiments, the contents of the channel could be living cells, assay reagents, sensing molecules, particles, or beads. For example, in cellular or biochemical assays, gases that inhibit respiration or metabolism, i.e., toxins can be studied along with other gaseous forms of cell signaling agents. Many different types of gases could be used including pure gasses atmospheric gas, as well as other combinations or types of gasses.

[0045] The configuration in the figure above serves to demonstrate a single illustrative embodiment of the present invention. Those skilled in the art could readily envision and implement other means including alternate manifold designs by which gasses could be brought into diffusive communication with the membrane surface as well as to construct arrays of sensors such as on a standard microplate pitch, for example, to simultaneously sense the gasses contained within the wells of a microplate.

C. Assembly with Mechanically Actuated Valve

[0046] In some embodiments, the present invention further comprises integrated fluidic valves using the membrane material. For example, Figures 9-11 illustrate the operation of a mechanically actuated valve according to the present invention. Figure 9 and 10 show cross sectional views of a microfluidic device 230 with an integrated valve at location 232. Fluid flows into inlet 104d of substrate assembly 118d through channel 106d covered by membrane 110d and exits at outlet 112d. The open position of valve 232 is indicated by 238a in figure 9.

[0047] Figure 10 is a cross sectional view 250 showing the valve in the closed position 238b. Valve 232 is actuated through a vertically force mechanically applied to the membrane by external actuator 234.

[0048] Figure 11 is a top view 260 showing the relative location of valve 232 along channel 106d of substrate assembly 112d. Valve 232 is closed when gas permeable membrane 110d and its bonding layer 236 are physically depressed by actuator 234 into channel 106d at position 232 stopping both fluid flow and chemical diffusion. The Channel depth Dc is chosen so that the membrane 110d and its bonding layer 236, can be depressed with an appropriate force so as to sealably contact the bottom of the channel. If the bonding layer is fabricated with a tacky substance such as a silicone adhesive, the valve will remain closed even after the actuation force is removed by sticking permanently to the bottom of the channel after it is actuated. If a reversible valve is desired, the bottom surface must release

spontaneously from the bottom of the channel once the actuation pressure is removed or else be bonded to the actuator and be opened when the actuator is retracted.

[0049] Figure 12 shows a top view 300 of a substrate 308 which contains an actuator post array 304 comprised of a plurality of raised actuator posts 306. Application of assembly 300 with the proper force to a suitably designed chip according to the present invention will cause simultaneous closure of all of the valves contacted by the actuator posts 306. Each individual valve in the array operates separately as is shown in figures 9-11 and when actuated by assembly 300, the individual vales are actuated as an array. Alignment pins 308 or other equivalent mechanical or optical means are provided to insure that the actuator posts 306 are physically aligned to the chip whose valves are to be actuated with the precision required to insure that the actuator posts make contact with their intended valve areas.

[0050] In a preferred embodiment, the actuator post array 304 is fabricated by etching a transparent glass substrate 302 everywhere but at the actuator post locations 306. Fabricating of actuator substrate 302 from an optically transparent material enables optical observation of the chip to occur simultaneously with valve actuation.

[0051] Figure 13 is a side view showing the profiles of the actuator posts. The actuator posts must be tall enough to insure that the valves will be completely closed when the substrate and valve array microfluidic device are compressed together with the appropriate force. If the bonding layer has an adhesive surface, the valves will stay closed even after actuator is separated from the microfluidic device.

[0052] Figures 14-16 illustrate alternate embodiments for actuating a plurality of valves according to the present invention. In these embodiments, an externally applied pressure is distributed to exemplary valves 330 and 332a-d via a pressure distribution manifold comprised of exemplary pressure access ports 342 and 344 and exemplary channels 324 and 326 disposed in a second microfabricated substrate 321.

[0053] Figure 14 shows a top view of a chip 320 to which exemplary externally controlled fluidic pressures, (e.g. pneumatic or hydraulic) P1 and P8 are applied to access ports 342 and 326 and distributed by channels 324 and 326 to valves 330 and 332a-d disposed at specifically designated valve locations on fluid channel 334 thus providing an actuation force at each designated valve location.

[0054] Sources for externally controlled pressures P1 and P2 may be pneumatic or hydraulic and may be coupled to the pressure distribution manifold by sealably connecting the external controlled pressure to access ports 342 and 344 in second substrate 321. Those skilled in the art will recognize that there exist many ways to form a sealed connection to the

exemplary access ports of figure 14-16 including the use of compressible gasket materials, compressible o'rings, or by fabricating substrate 321 from a compressible material, all of which sealable means provide for removable or reusable sealed connections. Compressible materials include but are not limited to rubbers, elastomers, fluoropolymers, and the like. In some embodiments, it may be preferable to provide a permanent seal in which case one skilled in the art would choose from various means available to achieve permanent seals such means including curable adhesives, pressure sensitive adhesive layers, solvent bonding, ultrasonic bonding, or other means of forming chemical or physical bonds.

[0055] Figure 15 shows a side view 340 of a structure comprising a first assembled substrate 118e fabricated from substrate material 101e, an exemplary fluid channel 334, exemplary access ports 346 (similar to access ports 102a and 104a of figure 6), and attached permeable membrane 110e. The assembly comprising first assembled substrate 118e, channel 334, exemplary access ports 346, and permeable membrane 110e is laminated to a second substrate 321. Exemplary rectangular access port 336 is included to show that access to the permeable membrane can be provided via the same process steps used to fabricate round access ports 342 and 344.

[0056] Figure 15 illustrates an embodiment 340 wherein the valve actuation access ports 342a and 344a are located on the side of chip opposite the exemplary fluid access port 346.

[0057] Figure 16 illustrates an embodiment 350 wherein the valve actuation access ports 342b and 344b are located on the same side of chip as the exemplary fluid access port 356.

[0058] In the embodiments shown in figures 14-16, it is possible for the pressure distribution channels to cross over fluid channels without collapsing them when pressure is applied, i.e., activating a parasitic valve, since the area of the valve as designed is much larger than the cross over area of exemplary pressure distribution channel 326 and exemplary fluid channel 334. This means that whereas the force applied to a designated valve is sufficient to cause the valve to close; this is not the case for a cross over where the force is not sufficient to cause a parasitic valve to close. An important aspect of the design and implementation of practical and useful valves is that the membrane is deflectable with a reasonable applied pressure. Parameters which affect the deflection pressure are the valve area, channel width and depth, membrane thickness, and membrane elastic modulus. The present invention provides sufficient flexibility with regard to these design parameters to enable useful and practical valves to be designed and implemented by those skilled in the

microfabrication engineering art.

[0059] Another important feature of these exemplary embodiments is that a single exemplary pressure distribution channel such as 324 may be used to actuate a plurality of exemplary valves such as 332a-d. This is because the force applied to every valve in steady state is solely a function of the area of the valve and not the number of valves. Another feature of these embodiments is that the locations of exemplary access ports 1-8 may be disposed with a port to port spacing matching that of a standard microplate well pitch so as to optimize compatibility with standard microplate laboratory instruments and automation.

III. Assembly Fabrication

[0060] Manufacturing of the assemblies of the invention may be carried out by any number of microfabrication techniques that are well known in the art. For example, lithographic techniques may be employed in fabricating glass, quartz or silicon substrates, for example, with methods well known in the semiconductor manufacturing industries. Photolithographic masking, plasma or wet etching and other semiconductor processing technologies define microscale elements in and on substrate surfaces. Alternatively, micromachining methods, such as laser drilling, micromilling and the like, may be employed. Similarly, for polymeric substrates, well known manufacturing techniques may also be used. These techniques include injection molding techniques or stamp molding methods where large numbers of substrates may be produced using, e.g., rolling stamps to produce large sheets of microscale substrates, or polymer microcasting techniques where the substrate is polymerized within a microfabricated mold. Two exemplary methods of fabricating the present invention are provided herein. It is to be understood that the present invention is not limited to fabrication by one or the other of these methods. Rather, other suitable methods of fabricating the present devices, including modifying the present methods, are also contemplated.

[0061] More specifically, fabrication and assembly of substrate assembly 118e and permeable membrane 110e are as taught in FIG 17-32. Second substrate 321 is preferably microfabricated from an optically transparent material and laminated to permeable membrane 110e as taught by FIG 28-32 or as otherwise known to those skilled in the art. Second substrate 231 may be fabricated from a hard material such as glass or plastic or from an elastomeric material such as silicone rubber. If the second substrate is fabricated from a hard material which is not gas permeable, access ports such as 336 can be provided to allow gas exchange between the interior of the channel and the external environment. Alternatively, gas exchange can be provided at a desired region by providing a venting channel and access

port in second substrate 321 with a connection to the external environment.

[0062] Figure 17 illustrates an exemplary process flow chart 360 for the fabrication of a microfluidic well plate assembly 610 such as 610a (FIG. 34, FIG. 35), 610b (FIG. 36, FIG. 37). A substrate 101 is provided in process 362 upon which a microfluidic structure is fabricated in process 364, which comprises a fabrication process 366 of channels 106 on a channel surface 103a of a substrate 101 as well as a fabrication process 368 of access ports 104, 112 between channel surface 103a and an access port surface 103b opposite the channel surface 103a, e.g. 106a, 103a, 103b, 101a, 104a, 112a (FIG 6). The fabricated substrate 118, e.g. 118a (FIG 6) is then singulated in process 370. The channel surface 103a of the singulated substrate 118 is then sealably attached in process 372 to a membrane 110, e.g, 110a (FIG 6). The access port surface 103b of the fabricated substrate 118 is then sealably attached in process 374 to well frame 612.

[0063] Figure 18 is a cross sectional view 400 of a starting glass substrate 101 for use in process 362. Process 364 of figure 17 is comprised of two sub-processes 366 and 368 for fabrication of channels and access ports in the substrate. Numerous methods for fabricating equivalent channels and ports such as chemical or plasma etching, milling with laser, ultrasonic or mechanical means, drilling with laser, ultrasonic, or mechanical means are known to those skilled in the art and can be substituted to obtain the equivalent result of step 364 some combinations of which may preferably combine or reverse the order of steps 366 and 368.

[0064] An exemplary set of steps to implement process 366 of figure 17 in which channels are fabricated in a substrate is shown in figures 19–24. Figure 19 shows a process 410 to apply masking material 412 to a surface, e.g. 103a (FIG6) of substrate 101. Figure 20 shows a step 420 to apply photoresist 422 to masking material 412. Figure 21 shows a step 430 to expose and develop photoresist 422 leaving areas of developed photoresist 434 and exposed masking material 432. Figure 22 shows a process step 440 to etch masking material 412 in exposed areas 432 forming an etch mask. Figure 23 shows a step 450 to etch areas of glass exposed by the formation of patterned etch mask 432 in step 440. Figure 24 shows a step 460 to strip etch mask material 422 and etch mask 412 from etched substrate 101.

[0065] An exemplary set of steps to implement process 368 of figure 17 in which the access ports are fabricated in the substrate are shown in figures 25–27. Figure 25 shows a step 470 to apply a sand blast mask 472 to surface, e.g. 103a (FIG6) of substrate 101 opposite area of etched channel 432, e.g. 103b (FIG6) leaving exposed substrate areas 474. Figure 26 shows a process step 480 to fabricate holes by sand blasting areas 482 exposed by sand blast

mask 472. Figure 27 shows a process step 490 to remove sand blast mask 472 leaving substrate 101 including channels 106 and access ports 482 forming substrate assembly 118. Access ports 482 are exemplary and form the basic structures for fluid inlet and outlet ports for use in numerous potential embodiments of the present invention, e.g. 104a, 112a (FIG 6).

[0066] Referring to figure 17, the substrate with channels and ports as fabricated in process 364 is then singulated in process 370. Numerous methods for singulating substrates are known to those skilled in the art such as dicing, sawing, laser cutting, scribing and breaking and can be substituted to obtain the equivalent result of process 370.

[0067] An alternate and potentially preferable process is to reverse the sequence of processes 370 and 372. In the alternate process the substrate is sealably attached to the membrane prior to singulation of the individual chip or chips from the starting substrate. Choice of a preferred membrane attachment and singulation process and sequence will depend on the size of the final chip relative to the size of the starting substrate, the substrate, bonding layer, and membrane materials, and the most cost-effective method to manufacture the final product.

[0068] The membrane is then sealably attached to the substrate in process 372 of figure 17. Numerous methods for sealably attaching adhesive films or bonding layers to substrates are known to those skilled in the art such as lamination processes utilizing pressure and heat, pressure and ultrasonic energy, pressure sensitive adhesives, plasma surface treatments such as oxygen or plasma in the presence of other pure gasses or gas mixtures, chemical surface treatments such as silanes, silicones and the like and such processes can be substituted to obtain the equivalent result of process 370. In addition methods for applying and patterning the bonding layers used in processes 372 and 374 are also known to those skilled in the art. Methods of applying bonding layers include but are not limited to thin or thick film lamination, spin coating, spray coating, dip coating, extrusion, co-extrusion, and chemical or vapor deposition or direct dispensing through an orifice. Methods for patterning bonding materials or bonding layers include but are not limited to photolithography of resist masks, photosensitive bonding materials, direct printing of bonding materials by ink jet, silk screen or etched stencils, or direct stamp transfer methods of bonding materials as well as application of bonding materials to surfaces treated by or assisted using isotopic or anisotropic plasma processes, finally direct dispensing of bonding materials through an orifice under automated computer control.

[0069] In a preferred embodiment, a layer of a photosensitive material with photo-cleavable terminating groups would be applied to the surfaces of the chip after fabrication of

the channels and/or the access ports. The photosensitive material would then be exposed directly or through a photo-mask to remove the photo-cleavable terminating groups in specifically desired areas. Subsequently the chip would be exposed to an agent that would bond selectively to only the photo-cleaved terminating groups. By repeating this exemplary process, a first agent could be applied only to areas where bonding to the membrane was desired and a second agent could be applied to the inside of the channels or access ports where compatibility with a particular assay or substance to be used in a specific application was desired.

[0070] An exemplary set of steps to implement process 372 of figure 17 by which the membrane is sealably attached to the substrate is shown in figures 28–30. Figure 28 shows a cross sectional view 500 of a starting membrane 110. Figure 29 shows a process step 510 to apply a bonding layer 512 to membrane 110. Figure 30 shows a process step 520 to bond membrane 110 and bonding layer 512 to fabricated substrate 118 by bringing applied bonding layer 512 into intimate contact with substrate 118 under the appropriate environmental conditions for the appropriate time. Process step 520 results in an assembled chip 542 comprised of substrate assembly 118 and membrane 110.

[0071] An alternate exemplary set of steps to implement process 372 of figure 17 by which the membrane is sealably attached to the substrate is shown in figures 31–32. Figure 31 is an alternative process step 530 in which bonding monolayers 532a and 532b are applied to the surfaces of membrane 110 and substrate 101, respectively. Figure 32 shows a process step 540 to bond membrane 110 to substrate 101 forming an assembled chip 542 having an interfacial bond 544 between monolayers 532a and 532b by bringing applied bonding monolayers 532a and 532b into intimate contact under the appropriate environmental conditions for the appropriate time.

[0072] Referring to figure 17, the singulated substrate with attached membrane 542 is sealably attached to a well frame in process 374. Numerous methods for sealably attaching a singulated substrate to a microplate well frame are known to those skilled in the art such as through the use of suitable epoxies, UV curable adhesives, silicones and the like and can be substituted to obtain the equivalent result of process 374. Well frames could be fabricated from many materials including metals such as aluminum or stainless steel and plastics such as polystyrene or polypropylene, acrylic, polycarbonate, and Topas in any shape or size, however there are two standardized and preferred embossments according to the present invention. These are embodiments wherein the size of the well frame (i.e., the outer dimensions of length and width and possibly the depth) is chosen to be consistent and

therefore compatible with those of standardized microscope slides and micro titer plates or microplates. Exact standard sizes are not specified herein since those skilled n the art are aware of specific reference documents which detail specific standard sizes for microscope slides and microplates as used in specific applications. It is noted that while some variation in "standard" sizes occurs from application to application, the standard sizes are generally consistent within a given application area.

[0073] Methods of bonding the membrane to the substrate include the use of a silane bond. Various techniques can be used to preclude the silanes from depositing in undesired areas. For example, the channels can be etched with a strong base such as sodium hydroxide; the silanes can be patterned so as to avoid channels with Teflon etch mask; or the silane layer can be applied by transfer printing so as to avoid deposition in the channels.

[0074] Alternatively, a pressure sensitive adhesive may be bonded to the gas permeable membrane. This can be accomplished, for example, by using a die cut adhesive backed gas permeable membrane that is then applied, for example, with an automatic labeling machine. Alternatively, a stamp transfer adhesive can be used. Shown below are typical platen press conditions used in applying a membrane comprising Teflon to the solid substrate. Typical molding temperatures range from 240° to 275°C (464° to 527°F) for Teflon® AF 1600 and 340° to 360°C (644° to 680°F) for Teflon® AF 2400.

Typical Platen Press Conditions

Substrate	Interface Temperature, °C (°F)	Pressure, psi	Dwell Time, min	Substrate Surface Preparation and Treatment
Aluminum	282 (540)	100	5	None, if Type C film is used
	293 (560)	100	5	Parker Bonderite 700 series*
Copper	282 (540)	100	3--5	Various treatments
Steel	293--304 (560--580)	100--300	5	Sandblast and degrease, phosphatized*
Stainless steel				None
	360 (680)	300	5	Dispersion primer of Teflon® --
	293 (560)	300	5	see paragraph above
Teflon® TFE	343 (650)	100	3--5	None

Nickel	282 (540)	100	5	None, if Type C film is used
Nickel Ceramics Nichrome	293 (560)	300	5	Dispersion primer of DuPont FEP
Nomex® nylon paper	282 (540)	100	5	Use Type C film Pre-dry Nomex® (at 121°C [250°F], 30 min)
Glass	296 (565)	10	10	Silane coupling agent**
Kapton® polyimide film	282 (540)	100	5	None, if Type C film is used

**Treating and phosphating chemicals are available from Oxy Metal Industries, 322 Main St., Morenca, MI 49056.

**Silane coupling agents are available from Union Carbide Corporation and Dow Corning

IV. Representative Plate Assemblies

[0075] Figure 33 shows a perspective view 600 of an industry standard 96 well microplate. Industry standard generally refers to relatively standard footprint, height, and well to well pitch. Industry standards for microplates have been created by the Society for Biomedical Screening for various types of microplates including 96, 384, and 1536 well microplates which have standardized well to well pitches of 9 mm, 4.5 mm, and 2.25 mm, respectively. Standards exist for other types of microplates as well. One aspect of the present invention is to provide compatibility with these standards and allow products fabricated according present invention to be used with industry standard dispensing, detection, laboratory automation, robotics and other processing equipment. Figure 33 shows the location of cross section 611 which will be referred to in Figure 34.

[0076] Figure 34 shows a cross sectional perspective view of microfluidic well plate assembly 610a along section 611 of figure 33. This figure illustrates the inherent compatibility of mating an assembled chip 542 according to the present invention with an industry standard microplate well frame 612 similar to the one shown in figure 33. Surface 103b of assembled substrate 188 is sealably bonded to microplate well frame 612 using a process such as 374 of FIG 17 and process steps such as described in FIG 38-41. The assembled chip is preferably bonded to the microplate frame by an adhesive system which seals the edges of each well to prevent fluid leakage and optionally with the structural elements of the well frame. The outer surface of membrane 110 of assembled chip 542 faces

the bottom of the Microplate well frame so as to allow convenient viewing of the chip from below.

[0077] Figure 35 shows a partial cut away perspective view 620 of the microfluidic well plate assembly 610a of figure 34. It should be noted that the present invention can be incorporated into any standard microplate that exists and it is expected that the invention will be able to be incorporated into future standards as well. Sample wells 614 are preferably positioned over access ports 622, e.g., 104a, 112a (FIG 6) providing means for fluid connection and flow through channel 106f formed between the surface 108f of fabricated substrate 118f and surface 109f of membrane 110f.

[0078] Figure 36 shows a partial cut away top view 630 of an alternate embodiment of a microfluidic well plate assembly 610b similar to microfluidic well plate assembly 610a but preferably having conically shaped wells to accommodate smaller fluid volumes of directing them to and from the access ports 642 and channels 106 on the chip. This embodiment is similarly incorporated into an industry standard microplate format.

[0079] Figure 37 shows a partial cross section 640 of microfluidic well plate assembly 610b. Conical wells preferably efficiently hold and direct small quantities fluids to and from assess ports 643 particularly when the diameter of the conical wells 642 are relatively matched to the diameter of access ports 643. Figure 37 shows that assembled chip 542 can be optionally sealably bonded to the peripheral structure of microplate well frame 632 as well as to the fluid wells to increase the rigidity and flatness of microfluidic well assembly 610b. Microplate well frame 632 can be fabricated from a molded plastic structure to form wells 643 and outer form and footprint of microplate frame 632. If necessary a relief structure, such as a groove, can be incorporated into interfacial surface of the well frame at interface 646 to allow for expansion of the adhesive during curing.

V. Fabrication of Plate Assemblies

[0080] An exemplary set of steps to implement process 374 of figure 17 in which the completed substrate is sealably attached to the well frame is shown in figures 38-41. Figure 38 shows a process 650 providing an assembled chip 542, comprising a fabricated substrate 118 and attached membrane 110. Figure 39 shows a process 660 providing a well frame 632 to be laminated to assembled chip 542. Figure 40 shows a process 670 to apply an adhesive 672 to well frame 632. Figure 41 shows a process step 680 to laminate assembled chip 542 to well frame 632 with applied adhesive layer 672 by bringing adhesive layer 672 and completed substrate 542 into intimate contact under the appropriate environmental conditions for the appropriate time.

[0081] The materials used in any step of the fabrication process 360 should preferably be selected and processed so as to be inert with respect to the ultimate intended application of the chip, e.g., for use in chemical, biochemical, and biological assays, performing chemical reactions, or other applications. In other words, the materials must not leach or otherwise contribute toxic substances or other contaminants into the channels, chambers, or access ports or wells in amounts that would affect proper operation of the specific intended product application.

[0082] The bonding adhesive is preferably chosen to be compatible with the materials comprising the chip and well frame as well as the assay reagents which might come in contact with the adhesive and should be applied to avoid contamination of access ports 482 or channels 106. This can be accomplished by using any number of commercially available adhesives and processes for applying and curing the adhesive that does not impact the form or function of the device in a significant way. Such adhesives and processes are generally known to those knowledgeable in the art. For example, light curable adhesives, epoxies, silane layers, silicone adhesives and photo patternable adhesives can be used. Adhesives can be applied by silk screening, photolithography, roll coating, ink jet printing, or stamp transfer processes. If necessary a relief structure, such as a groove, can be incorporated into interfacial surface of the plastic well bottom at interface 7 to allow for expansion of the adhesive during curing.

V. Well frame assembly

[0083] Figure 42 is a perspective view 700 of a re-usable well frame assembly for sealably mounting, de-mounting and operating an assembled chip 542. Well frame assembly 700 is preferably fabricated with wells located on a standard microplate pitch such as 9 mm for a standard 96 well plate, 4.5 mm for a 384 well plate, 2.25 mm for a 1536 well plate and so on for other present and future standard plates. The assembled chip 542, having access port locations, e.g. 104a, 112a (FIG 6) preferably matching the well locations of well frame assembly 700 is sealably mounted to well frame body 710 by o'rings 724 and secured by assembly clamp 726. Well frame assembly 700 is designed to be compatible with and viewable by standard laboratory microscopes while allowing direct access to membrane surface 110i. Additionally well frame assembly 700 provides flexibility and compatibility with existing fluid handling equipment such as fluid dispensers and pipetting systems by providing access to a plurality of wells 728 on the surface of well frame body 710 opposite membrane 110i. Finally, well frame assembly 700 also provides access to and control of each individual well thus providing the flexibility to develop new assays or other applications

of assembled chip 542.

[0084] A plurality of wells 728 is disposed in well frame body 710 to hold reagents and direct them to and from associated chip access ports and channels within assembled chip 542. A plurality of connectors 712 are disposed within well frame body 710 to direct sources of externally generated and controlled pneumatic or hydraulic pressure to the plurality of wells 728. Tapered plugs 714 or equivalent alternative means is provided to seal the well entrances allowing the externally controlled pressure directed through connectors 712 to wells 728 to build up within the wells and provide a driving force for flow within the channels of assembled chip 542. Those skilled in the engineering arts will readily recognize that alternative designs exist by to implement well frame assemblies with functions equivalent to those of well frame assembly 700 and the exemplary design described herein should not be interpreted as a limitation of the present invention. For example, an alternate embodiments, of well frame assembly 700 could comprise plastic molded components or sealing elements and could be designed for one time disposable use or for reusability.

[0085] In the absence of an externally generated and controlled source of pneumatic or hydraulic pressure, flow in channels within the chip is preferably driven by head pressure generated by gravitational forces on the fluid columns in the wells. The height of the fluid in the wells determines the head pressure applied to the wells and this can be determined by the volume of fluid dispensed into the wells. Preferably, means to provide a uniform meniscus on the fluid in the wells is provided to minimize flow variations caused by well to well meniscus shape variations.

[0086] The methods and technologies illustrated for control of flow control in the re-usable well frame assembly 700 in figure 42 are scalable from the microscope slide to the microplate plate formats shown in figures 34-37.

[0087] When not engaged in well frame assembly, flow in the chip is driven by head pressure generated by gravitational and surface tension forces on the fluid columns in the chip access ports. External packaging such as been described previously may be used to increase the height of the fluid column to generate additional head pressure. Additionally, portable pressure reservoirs may be included in either the chip packaging or well frame assembly to accommodate various application requirements.

VI. System for Operating a Microfluidic Chip

[0088] Figure 43 shows an architectural block diagram for a system 740 for operating a microfluidic chip and associated reagents 746 comprising a chip controller 742 and a chip reader and associated software 744. Chip controller 742 communicates with a microfluidic

chip and associated reagents according to the present invention 746 through fluidic control interface 748 which may preferably comprise pneumatic, hydraulic, electronic, mechanical, or optical means or a combination of any of the afore mentioned control modalities. Chip reader and associated software 744 communicates with a microfluidic chip and associated reagents according to the present invention 746 through interface 749 which may preferably comprise pneumatic, hydraulic, electronic, mechanical, or optical means or a combination of any of the afore mentioned control modalities.

[0089] Chip controller 742 controls fluid flow in microfluidic chip and its associated reagents 746 by supplying regulated and controlled sources of pressure or flow through interface 748. Either constant pressure or constant flow control is possible by providing a suitable configuration for the desired control embodiment. In a constant pressure control embodiment, the externally controlled source would preferably provide a source of constant pressure to the inlet or outlet wells and thus control flow in the channels. In a constant flow control embodiment, the externally controlled source would preferably provide a source of constant flow to the inlet or outlet wells and thus control flow in the channels. In a hybrid flow control embodiment, the externally controlled source would provide a hybrid of constant pressure and constant flow control to the inlet or outlet wells and thus control flow in the channels. This includes a hybrid controller capable of switching back and forth between constant pressure and constant flow modes. Either constant pressure, constant flow, or hybrid flow controllers can be operated in the "closed loop mode" if equipped with means to measure a signal proportional to the flow to be controlled and use this information as a feedback signal for servo control of the flow of fluid in a channel. Methods for design and fabrication, and configuration of flow controllers providing functions equivalent to or similar to those described herein are known to those skilled in the art.

[0090] Constant pressure control is preferred when the fluidic resistance of the microfluidic device is in a high enough range so that the pressure controller is capable of providing the desired degree of precision and accuracy of pressure regulation at the operating pressure. In cases where the fluidic resistance of the microfluidic device is relatively low, constant flow control is the preferred method of operation. In other cases, a hybrid between constant pressure and constant flow may be a preferred method of operation.

[0091] In a preferred embodiment, chip controller 742 comprises a computerized valve controller such as those commercially available from Automate Scientific Corp., an accurately regulated source of gas, and means for sealably interconnecting the pressurized gas source and the valve controller to chip 746. The regulated source of gas is preferably

designed to provide accuracy and precision of better than 0.01 PSI in the range of -10 to + 10 PSI so as to provide controlled flow velocities in the range of 0 to 1 meter per second with a precision of better than .1 mm/sec and preferably better than 10 micron per second and more preferably better than 1 micron per second.

[0092] In a second preferred embodiment, chip controller 742 comprises a computerized valve controller such as the ValveLink8 available from Automate Scientific, a precisely controlled syringe pump such as is commercially available from Cavro Scientific Corp., and means for sealably interconnecting the syringe pump and the valve controller to chip 746. The syringe pump is preferably designed to provide the desired accuracy and precision of the flow required in an actual operation of the microfluidic chip. Since flow rates in microfluidic devices are usually in the range of picoliters to nanoliters per second, the syringe pump should preferably be configured to provide a flows controlled to precision of 10% or better or preferably 1% or better or even more preferably 0.1% or better in certain applications so as to provide flow velocities in the range of 0 to 1 meter per second with a precision of better than .1 mm/sec and preferably better than 10 micron per second and more preferably better than 1 micron per second.

[0093] In a preferred embodiment, the system for operating a microfluidic chip and associated reagents 740 described above would be used to perform an assay selected from the group including but not limited to cellular, biochemical, and chemical assays. Assays of these types are preferably quantified by detection of a measurable change in an observable property of the assay in response to a specific stimulation or set of stimuli. For example, detection schemes can preferably involve readouts of changes in properties of the assay selected from a group including but not limited to bulk absorbance, transmission, turbidity, reflection, fluorescence, fluorescence lifetime, luminescence, refractive index, or imaging modalities such as the afore mentioned properties and other properties capable of being visualized with optical imaging technologies including morphology and relative position.

[0094] Optically transparent and low fluorescence background materials are preferably used in the construction of the microfluidic chip and formulation of the associated reagents 746 to enable the collection of optical data in modalities including but not limited to fluorescence, luminescence, time resolved fluorescence, fluorescence polarization, absorbance, and the like. Instruments for detection include but are not limited to point reading as well as imaging systems with single or multi wavelength measurements from ultra violet to infrared wavelengths with detectors ranging from photodiodes to avalanche photo diodes, to Photomultiplier tubes, to charge coupled devices (CCDs), enhanced CCDs, and

cooled CCDs. Imaging detectors have the advantage of being able to read out the entire sample field of view quickly whereas point readers can have higher resolution and contrast. The choice of observable properties and detection modes should not be considered to be limited by the above description since many different observable properties and detection schemes are known to those skilled in the art.

[0095] In an exemplary preferred embodiment of an assay and detection method, the observed property for the assay would be a change in binding of a ligand to surface receptors of living cells. The detection method would be a change in the fluorescence polarization levels of the signal received from a fluorescently labeled ligand within a channel of a microfluidic chip fabricated according to the present invention. The relatively thin depth of a channel within a microfluidic device according to the present invention results in relatively low fluorescence background being generated from substances within the channel compared to similar measurements in microplates. This allows fluorescence polarization measurements to be made as a direct readout of molecular binding within the channel. For example in one preferred embodiment, a labeled natural ligand for a cell surface receptor would be displaced by a test compound if the test compound had a similar or higher affinity for the receptor than did the natural ligand. Higher fluorescence polarization in the vicinity of the cells indicates a lower degree of displacement of labeled natural ligand. Conversely, as more and more labeled ligand is displaced, the observed fluorescence polarization decreases. Fluorescence polarization measurements in a relatively thin microfluidic channel potentially enables cell surface receptor and other similar binding assays to be carried out without the use of confocal detection methods which might be necessary in non-microfluidic formats.

[0096] In a preferred embodiment, images acquired from the microfluidic chip during an assay by a device such as a microscope and CCD camera would be analyzed using computerized image analysis software. For example, images acquired before, during, and/or after an assay is performed, in a preferable order, could be compared with each other using computer algorithms to quantify the changes in the observed property selected to read out the assay. For example in a first embodiment, simple subtraction of images of the observable property taken before and after an assay could preferably be used to quantitatively read out the assay results. Alternatively, in a second embodiment, autocorrelation algorithms could preferably be used to provide a quantitative indication of the extent of specifically measurable changes in the observable property during the assay.

[0097] Figure 44 is an architectural block diagram750 of a system for operating a chip according to the present invention in a automated laboratory environment. Chip

controller and reader 757 communicate with robot controller 758 through interface 752a enabling the chip controller and reader to be controllably integrated into an automated laboratory environment. Microfluidic well plate assembly, 610, comprising a microfluidic chip according to the present invention packaged in an industry standard microplate format, e.g., (FIG 33-FIG 37) designed to be compatible with industry standard physical conventions provides an interface 755b to standard laboratory robotics 759. An aspect of the present invention is to intentionally provide compatibility with laboratory robotic standards enabling products made according to the present invention to be readily used in conjunction with industry standard fluid dispensing, detection, and other robotics and automated processing equipment.

[0098] Communication between chip controller and reader 757 and pressure manifold 754 takes place via interface755a. Manifold 775 is mechanically aligned and sealably mounted to packaged chip 610 and distributes the pneumatic, hydraulic, electronic, mechanical, or optical signals to and from the chip controller and reader 757 to their intended destinations on packaged chip 610. Manifold 777 configured to be structurally compatible with standard laboratory robotics 759 by conforming to applicable industry standards such as form factor, physical access, and compatibility with laboratory automation systems.

VII. Embodiments having a Microscope Slide Format

[0099] Figure 45 is a plan view of a microscope slide sized substrate 760 having access ports 764 in standardized locations on substrate 762. Substrate 762 is preferably configured as a exemplary standard nominal 25 mm X 75 mm microscope slide with access ports 770a-770m on a row pitch 772 corresponding to an industry standard microplate well pitch, preferably 9 millimeters for a 96 well plate, 4.5 millimeters for a 384 well plate, etc. Conformance to an industry standard microplate well pitch by row or along the long dimension of substrate 764 enables compatibility with multi-tipped fluid pipetters, pin tools, or automated dispensers or other standardized laboratory fluid handling equipment configured with linear microplate well spacing formats. Access ports 766a and 766b are shown with a column pitch 768. In a first embodiment, column pitch 768 is preferably chosen for most convenient use with standard 25 mm X 74 mm microscope slide substrate 762 and compatibility with fluid handling equipment configured with linear microplate well spacing formats. In a second preferred embodiment, column pitch 762 is chosen to correspond to a standard microplate well pitch, preferably 9 millimeters for a 96well plate, 4.5 millimeters for a 384 well plate, etc. Conformance to an industry standard microplate well pitch by row and column or along the long and short dimensions of substrate 764

enhances compatibility with multi-tipped fluid pipetters, pin tools, or automated dispensers or other standardized laboratory fluid handling equipment configured in a partial microplate format.

[00100] Figure 46 illustrates a cut-away plan view 774 of exemplary 2 port 776a and 4 port 776b standard unit cells having standardized access ports 764 located on substrate 762 with column spacing 768 and row spacing 772 as shown in figure 45. View 774 illustrates the idea of a standard unit cell that can be preferably designed to perform a specific function and then replicated and placed so as to mate with other standardized access port locations on substrate 762. In this example, there are two standard unit cells, 776a with two ports and the 776b with uses four ports illustrating that it is also possible to design microfluidic circuits with multiple standard unit cell types

[00101] Figure 47 is a plan view 780 of three replications of an exemplary unit cell 776c having three channels merging into one (a 3-1 combiner structure), which has been placed to optimally utilize the standard access ports locations of substrate 762 shown in figure 45. In view 780 of figure 47, substrate assembly 118j is fabricated with access ports and channels according to the present invention and access ports in the standardized locations of substrate 762 in figure 45. One of the standard cells is flipped horizontally to better utilize the access ports. This enables the use of multi-tipped pipetters, pin tools, or other standardized laboratory equipment to deliver fluid to the assess ports.

[00102] Figure 48 is an expanded view 790 of 3-1 combiner standard unit cell layout 776 which utilizes the standard access ports locations of substrate 762 shown in figure 45. View 790 shows substrate assembly 118j having standard unit cell 776d with a 3-1 combiner structure connected to standard access port locations from figure 45. Three input channels 792, 794, and 796 connected to standardized access ports 770a, 770b, and 770c, respectively, are combined at junction point 797 into one output channel 798 connected to standardized access port 770d. There are numerous preferred uses for this structure in chemical, biochemical, and cellular assays. Those skilled in the art will be readily able to design such assays using this of variants of this structure using the teachings of the present invention.

VIII. Embodiments having a Standard Well Plate Format

[00103] Figure 49 shows a plan view 800 of an exemplary array 804 of standard unit cells 820 replicated 96 times in an 8 row by 12 column arrangement in an industry standard 384 well format, each unit cell having up to 4 access ports. Those skilled in the art will recognize that it is possible to populate standard unit cell arrays for industry microplate formats with standard unit cells having a number of wells that are evenly divisible into the

total number of wells in the a given microplate format. For example, preferred numbers of wells for standard unit cells in all microplate formats can include but are not limited to 2 wells, 4 wells, 8, wells, 16 wells, 32 wells, 64 wells, 96, and 384 wells.

[00104] Figure 50 is a view 820 of exemplary 4 port standard unit cells 830 and 8 port standard unit cell 832 each with alternative channel network configurations, any of which being suitable for placement into a standard unit cell array such as in view 800 shown in figure 49. Unit cell 822 has two intersecting channels from ports 1-4 and 2-3. Unit cell 824 has channels from ports 1,2, and 3 to port 4. Unit cell is an H channel structure with channel connections between ports 1-2 and 3-4 and a bridge between 1-2 and 3-4. Unit cell 828 is comprised of two 2-port sub unit cells with channel connections between 1-2 and 3-4. Unit cell 832 is an 8 port unit cell with three H equivalent structures connected in parallel to allow distribution of a common reagent from wells 7 and 8 to assay regions within a portion of channels 1-2, 3-4, and 5-6. The operation of this structure is discussed in figure 68.

[00105] The well to well spacing or well pitch of the standard unit cells is preferably designed to match industry standard microplate well pitches including but not limited to 96, 384, and 1536 well formats. External form factors and well pitch are preferably designed to be consistent with industry standard microplate formats and packaging, e.g., 610a, 610b (FIG 34-36), to enable microfluidic well plates according to the present invention to be compatible with standardized fluid handling equipment.

[00106] Figure 51 illustrates a standard unit cell array of 4 port standard cells 844 in an industry standard 96 well format 840, array 844 comprising 24 repetitions in a 4 row by 8 column layout of unit cells comprising a 3-1 combiner structure fabricated in substrate assembly 118k.

[00107] Figure 52 illustrates a standard unit cell array of 4 port standard cells 854 in an industry standard 384 well format 850, array 854 comprising 96 repetitions in a 8 row by 12 column layout of unit cells comprising a 3-1 combiner structure fabricated in substrate assembly 118l.

[00108] Figure 53 illustrates a standard unit cell array of 4 port standard cells 864 in an industry standard 1536 well format 860, array 864 comprising 384 repetitions in a 16 row by 24 column layout of unit cells comprising a 3-1 combiner structure fabricated in substrate assembly 118m.

IX. Routing Embodiments

[00109] Figure 54 is a plan view 870 of unit cell array 874 having exemplary routing networks 878a and 878b for distributing two common reagents 880a and 880b, respectively,

to each site in exemplary array 874 of 96 standard unit cells 876 874 in an industry standard microplate format similar to the exemplary array 804 of unit cells 820 described in figure 49. Reagent 1 is distributed to the unit cells from left to right whereas reagent 2 is distributed from right to left. Offsetting the reagent routing lines allows access to every unit cell.

[00110] Figure 55 is a partial expanded view 890 of the exemplary network of channels 878a and 878b shown in figure 54 for distributing two common reagents 880a and 880b, respectively, to exemplary standard unit cells 876 within a standard unit cell array (wherein test compound is denoted with "C", and waste, "W"). Distribution networks 878a and 878b supply reagents 880a and 880b, respectively, to each unit cell in an array of four exemplary unit cells 876 each containing a 3-1 combiner structure. Unit cell 876 contains one well 892 for test compound and one well 894 for waste. Connector channels 896a and 986b couple reagents 880a and 880b into merge point 898 of standard unit cell 3-1 combiner structure 876 from reagent distribution networks 878a and 878b, respectively.

[00111] This layout demonstrates that global routing of two reagents can be accomplished by routing one reagent from the left side of the plate and one from the right side. The compound wells can be accessed with standard dispensing equipment since they are located on the standard well pitch and can be designed to hold volumes within the working range of conventional dispensers, i.e., 1-2 microliters. Waste wells are provided at each site to minimize back pressure although extra reagent wells could be include at each unit cell site.

[00112] Figure 56 illustrates an exemplary routing network 900 having a ring configuration for distribution of a common reagent within an 8 port standard unit cell. Using exemplary network 900, reagents such as biological cells, beads, particles and other substances used in chemical, biochemical, or biological assays can be distributed from a common source well to multiple destination wells by increasing the pressure in the source well relative to that of the destination wells.

[00113] Figure 57 shows an exemplary linear channel network 910 within an 8 port standard unit cell that is functionally equivalent to the ring channel network of figure 56. Linear configuration 910 affords a perspective from which one can visualize how the amount of reagent distributed to each well of the unit cell from the source well can be controlled by controlling the pressure applied to each well by an external source. The fluid path from well 1 to well 8 is comprised of two parallel paths, one through wells 1-2-4-6-8 and the other through wells 1-3-5-7-8. All other parameters being equal such as channel fluid resistance as a function of channel length, a pressure applied to well 3 would be distributed equally along

both parallel flow paths if an external pressure applied to each of the corresponding well pairs, 2-3, 4-5, and 6-7 were adjusted to a pressure at which the flow into the wells from the channels was zero. In this case flow in the channel would proceed from well 1 to well 8 with no flow into wells 2-7. Transport of substances from the channels to the wells would occur by diffusion only. Transport by diffusion could be utilized in numerous assays such as cell migration assays where it was desired to inject a very small and controllable amount of reagent thereby inducing a concentration gradient. By adjustment of the external pressure applied to each well, flow could be set to a range of desired levels including zero. Utilizing controlled pressure at the destination wells, controlled distribution of reagents such as cells, beads, or particles from a common source well to a designated destination well could be accomplished.

[00114] Figure 58 illustrates an 8 port standard unit cell 920 in a microplate format with a ring channel network. Configuration 920 is fluidically equivalent to configuration 910 of figure 57 and configuration 900 of figure 56 with the exception that the path length between all of the wells is not equal as drawn. However, the distances between wells can be equalized simply adding extra path length, for example, between channels 1-2 and 7-8 by inserting a serpentine cannel structure of the appropriate length. Configuration 920 with equal channel lengths is capable of providing the function of configuration 910 of figure 57 or configuration 900 of figure 56 and is also compatible when configured as an 8 port standard unit cell in industry standard microplate formats.

[00115] Figure 59 shows a 4 port standard unit cell 930 in a microplate format with a ring channel network. Configuration 930 has the unique advantage that the all of the path lengths between adjacent wells are equal as drawn in figure 59 it is usable as a standard unit cell in an industry standard microplate format. In a preferred embodiment of a cell migration assay, a common reagent such as a chemoattractant is dispensed into well 1. Cells are dispensed into wells 2 and 3 and allowed to attach to the surface. Test compounds 1 and 2 designed to target the biological target to be tested are then dispensed into wells 2 and 3 respectively. Pressure P1 is applied to well 1 and pressure P1/2 is applied to wells 2 and 3 insuring that no flow occurs between the channels and wells. Cell migration can then occur in the concentration gradient formed at the junction of the channels and wells 2 and 3. In configuration 930, the common reagent is used by two wells saving common reagent. If the same assay were carried out in configuration 910 in figure 57, one common reagent would be used by 6 wells providing even more savings. One skilled in the are can envision designing protocols by which cells and reagents could be initially dispensed into a single well and then

distributed to the other test wells within each unit cell. Larger unit cells would potentially provide higher utilization and larger savings in common reagents. However, one expects that there is a preferred configuration wherein the best balance is achieved between savings of common reagents and complexity of flow control.

[00116] Figure 60 shows a 4 port unit cell with an H channel network configuration 944 in a microplate format 940. Table 2 provides an exemplary protocol for loading cells into an assay region of the channel bridging channels 1-2 and 3-4 of H structure 940 and running a cell migration assay. The advantage of this cell migration assay configuration is that the gradient in the assay region of the bridging channel would remain constant during the assay. The protocol assumes the use of a multi-well pipetter compatible with industry standard microplate formats and therefore carries out each operation on all wells simultaneously. Those skilled in the art will readily recognize that the exemplary protocol shown in table 2 below can be extended to accommodate other assays including but not limited to chemical, biochemical, and cellular assays with cells, beads, and other reagents.

#	Step	Well 1	Well 2	Well 3	Well 4	Flow
1	Dispense media	(+) media	(+) media	(+) media	No-op	No
2	Prime, de-bubble	P(prime)	P(prime)	P(prime)	0	Yes
3	Remove media	(-) media	(-) media	(-) media	(-) media	No
4	Dispense cells	(+) cells	(+) media	(+) media	(+) media	No
5	Load cells	P1(load)	P2(load)	P3(load)	0	Yes
6	Remove cells	(-) cells	(-) media	(-) media	(-) media	No
7	Add media	(+) media	(+) media	(+) media	(+) media	No
8	Stop flow	P(stop)	P(stop)	P(stop)	P(stop)	No
9	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No
10	Remove media	(-) media	(-) media	(-) media	(-) media	No
11	Add compound	(+) cpd	(+) media	(+) media	(+) media	No
12	Load compound	P1(load)	P2(load)	P3(load)	(+) media	Yes
13	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No
14	Remove media	(-) media	(-) media	(-) media	(-) media	No
15	Add chemoattractant	(+) chemo +cpd	(+) media	(+) cpd	(+) media	No
16	Run assay	P(assay)	No-op	P(assay)	No-op	Yes

Table 2; Cell migration assay protocol for use with the H structure 940 of figure 60.

[00117] Figure 61 shows a star channel network 950 for distribution of a common reagent from source well A to destination wells 1-8 within a multiple port standard unit cell. In star configuration 950, a common reagent such as cells, beads, or particles is dispensed to well A and pressure is applied causing fluid to flow from source well A to destination wells 1-8. An advantage of this configuration is that it is not necessary to apply external pressures to wells 1-6 in order to distribute reagents equally to all destination wells.

[00118] Figure 62 illustrates a linear channel network 960 within an 8 multiple port standard unit cell with function equivalent to the star channel network 950 of figure 61. The fluidic resistance between wells 3-6 and the center point between well 1 and 8 can be designed to be equal by varying channel lengths, widths, and depths accordingly. The pressure at the mid position between well 1 and 8 can be controlled by controlling the pressures (positive or negative) applied to well 1 and well 8.

[00119] Figure 63 shows a multiple port standard unit cell 970 in a microplate format with a star channel equivalent network. Configuration 970 is fluidically equivalent to configuration 960 and it is therefore compatible with use as an 8 port standard unit cell in microplate compatible formats.

[00120] Figure 64 illustrates an exemplary serial channel network 980 for distributing a common reagent to a plurality of single channel standard unit cells 982a-982d within an 8 port standard unit cell in an industry standard microplate format. Isolation valves 986a-986c shown in the open position allow a common reagent, including but not limited to cells, beads, or particles, to be dispensed into a common source well, in this case well 1. Flow, driven by a pressure applied to the source well through the network, and thereby distributes the common reagent to an assay region of each standard unit cell channel 982a-982d.

[00121] Figure 65 shows exemplary standard unit cell 980 of figure 64 with the isolation valves 986a-986c in the closed position 990 and the common reagent, in this example cells, beads, or particles 992, distributed to and trapped within the assay region of each unit cell in the plurality of unit cells 982a-982d.

[00122] Table 3 provides an exemplary protocol for loading and distributing cells to an assay region of each standard unit cell channel 982a-982d of exemplary unit cell 980 and running a cell migration assay. The protocol assumes the use of a multi-well pipetter compatible with industry standard microplate formats and therefore carries out each operation on all wells simultaneously. Those skilled in the art will readily recognize that the exemplary protocol shown in table 3 can be extended to accommodate other assays including but not limited to chemical, biochemical, and cellular assays with cells, beads, and other reagents.

#	Step	Well 1	Well 2	Well 3	Well 4	Flow	Well 7	Valve
1	Dispense media	(+) media	(+) media	(+) media	No-op	No	No-op	Open
2	Prime, de-bubble	P(prime)	P(prime)	P(prime)	0	Yes	0	Open
3	Remove media	(-) media	(-) media	(-) media	(-) media	No	(-) media	Open
4	Dispense cells	(+) cells	(+) media	(+) media	(+) media	No	(+) media	Open
5	Load cells	P1(load)	P2(load)	P3(load)	P4(load)	Yes	0	Open
6	Remove cells	(-) cells	(-) media	(-) media	(-) media	No	(-) media	Open
7	Add media	(+) media	(+) media	(+) media	(+) media	No	(+) media	Open
8	Stop flow	P(stop)	P(stop)	P(stop)	P(stop)	No	P(stop)	Close
9	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No	P(stop)	Close
10	Remove media	(-) media	(-) media	(-) media	(-) media	No	(-) media	Close
11	Add compound	(+) cpd	(+) media	(+) cpd	(+) media	No	(+) cpd	Close
12	Load compound	P1(load)	0	P3(load)	0	Yes	P7(load)	Close
13	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No	P(stop)	Close
14	Remove media	(-) media	(-) media	(-) media	(-) media	No	(-) media	Close
15	Add chemoattractant	(+) chemo +cpd	(+) cpd	(+) chemo +cpd	(+) cpd	No	(+) chemo +cpd	Close
16	Run assay	P(assay)	No-op	P(assay)	No-op	Yes	P(assay)	Close

Table 3; Cell migration assay protocol for serially linked structure 980 of figure 64.

[00123] Figure 66 illustrates an exemplary serial channel network 1000 for distributing a common reagent to multiple 2-1 channel unit cells within an 8 port standard unit cell in a microplate format. Isolation valves similar to valves 986a-896c of figure 64 are shown in the open position. The method of operation and assay protocol is similar to that of exemplary unit cells 980 and 990 of figures 64 and 65 with the difference that the 2-1 unit cell configuration allows the formation of a gradient down the axis of the assay regions of the channels bridging wells 2-3 and 5-7, e.g., (FIG 69-78).

[00124] Figure 67 shows a plan view 1010 of the standard unit cell of figure 66, with the isolation valves in the closed position and a common reagent distributed to and trapped within the assay region of each 2-1 channel unit cell within an industry standard microplate format.

[00125] Figure 68 illustrates an exemplary embodiment 1020 of a standard unit cell comprised of H equivalent structures with an integrated parallel network for distribution of a

common reagent to the assay region of each of three unit cells within an 8 port standard unit cell in a microplate format. Common reagents are loaded into wells 7 or 8 and injected into assay region 982a-982c by the application of pressure between wells 7 and 8. The fluid resistance of devices 1-2, 3-4, and 5-6 and interconnect channels 1022a-1022c are designed so as to cause the fluid flowing in 1022c to split equally between devices 1-2. 3-4, and 5-6.

[00126] Table 4 provides an exemplary protocol for loading and distributing cells to an assay region of each standard unit cell channel 982a-982d of exemplary unit cell 1020 and running a cell migration assay. The protocol assumes the use of a multi-well pipetter compatible with industry standard microplate formats and therefore carries out each operation on all wells simultaneously. Those skilled in the art will readily recognize that the exemplary protocol shown in table 4 can be extended to accommodate other assays including but not limited to chemical, biochemical, and cellular assays with cells, beads, and other reagents.

#	Step	Well 7	Well 1	Well 2	Well 8	Flow
1	Dispense media	(+) media	(+) media	(+) media	No-op	No
2	Prime, de-bubble	P7(prime)	P1(prime)	P2(prime)	0	Yes
3	Remove media	(-) media	(-) media	(-) media	(-) media	No
4	Dispense cells	(+) cells	(+) media	(+) media	(+) media	No
5	Load cells	P7(load)	P1(load)	P2(load)	0	Yes
6	Remove cells	(-) cells	(-) media	(-) media	(-) media	No
7	Add media	(+) media	(+) media	(+) media	(+) media	No
8	Stop flow	P(stop)	P(stop)	P(stop)	P(stop)	No
9	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No
10	Remove media	(-) media	(-) media	(-) media	(-) media	No
11	Add compound	(+) media	(+) cpd	(+) media	(+) media	No
12	Load compound	P7(load)	P1(load)	0	P8(load)	Yes
13	Incubate	P(stop)	P(stop)	P(stop)	P(stop)	No
14	Remove media	(-) media	(-) cpd	(-) media	(-) media	No
15	Add chemoattractant	No-op	(+) cpd	(+) chemo + cpd	No-op	No
16	Run assay	0	P(assay)	P(assay)	0	Yes

Table 4; Cell migration assay protocol for the H equivalent structure 1020 of figure 68.

[00127] Those skilled in the art will readily recognize that the exemplary protocol shown in table 4 can be extended to accommodate other assays including but not limited to chemical, biochemical, and cellular assays with cells, beads, and other reagents.

[00128] Those skilled in the art should readily recognize that unit cells such as those depicted and explained in figures 45-68 can be arranged, re-configured, and modified to implement a large variety of proffered embodiments to support many types of chemical, biochemical, and biological assays in industry standard microplate and microscope formats as well as in non-industry standard formats. The illustrative discussions provided herein should be construed not as limitations to the application of the present invention but as means to convey an understanding of the potential utility and many other potential applications possible of the present invention.

X. Applications

[00129] Embodiments of the present invention can be applied in numerous fields including basic biological science, life science research, drug discovery and development, as well as miniaturized chemical reactions such as DNA synthesis, protein synthesis, combinatorial chemistry, and general chemical synthesis.

[00130] There are several key needs in pharmaceutical drug discovery and development that drive the development of more efficient and powerful methods and tools for drug screening and testing. Methods and tools that provide highly relevant biological data are needed as early as possible in the discovery and development process to both eliminate drug candidates with inferior properties while identifying drug candidates with superior properties. Development and successful deployment of such methods and tools could ultimately provide lower drug candidates failure rates in clinical trials, reduce the number of post market release drug withdrawals, and reduce the cost of adverse side effects of drugs on the market. The net result would be to reduce the cost and time to develop higher quality drugs for use in therapies and as cures for diseases.

[00131] Cellular assays are becoming increasingly used because cells can provide more comprehensive and relevant data compared to some biochemical assays. Assays on primary cells from animal or human tissue are desirable since responses obtained from actual healthy and diseased tissue provide the ultimate target for testing drug candidates. However, primary cells are in limited supply since cells from both living sources and cadavers are precious, scarce, and costly to obtain and maintain. The net result is that assays using primary cells are relatively expensive compared to cultured or clonal cell lines and yet there is a need to large numbers of assays with primary cells. In order for more primary cell assays to be done within constrained budgets there is a need to reduce the cost per primary cell assay. The present invention reduces the cost per primary cell assay by providing methods and tools for extracting more information from fewer cells compared to other assays known in the prior

art.

[00132] Cellular assays or (i.e., live cell assays) are assays in which living cells play an integral role in the detection of bio-molecular interactions between the cells under investigation and the surrounding extra cellular environment or a specific biomolecule in the extra cellular environment. Bio-molecular interactions can be of numerous types including but not limited to ligand-receptor interactions, cell membrane interactions, protein-protein interaction, enzymatic interactions, nucleic acid interactions or nuclear receptors. Live cell assays can involve the interactions between cells as readout devices with specific biomolecules as well as with other cells. In live cell assays, changes in cell observable properties are detected in response to external stimuli or test drug additions. Many different observable properties can be detected using a variety of conventional detection schemes. Detection schemes can involve readouts of absorbance, fluorescence, luminescence, in the bulk or imaging modalities as well as readouts of changes in cell morphologies and the movement of cells across barriers such as membranes and gels.

[00133] In biological and drug discovery research many different types of cell based assays are performed. However, cell based assays generally require that the cells are seeded into a test environment e.g., a microplate well and then are given time to adjust to their new environment. For example, adherent Chinese hamster ovary CHO cells require a re-adjustment time during which they attach to the surface of the wells and then form a confluent monolayer. This generally takes about 12-24 hours. Non-adherent cells can also be used in cell based assays and these types of cells also require time to adjust to a new environment. In order to produce high quality and reliable data, the types of cell based assays generally require that the cells used in the assay are healthy and function more or less as they would in their native environment throughout the assay. Assay times can range from a few hours to a few days. Therefore, cell based assays can require preparative and assay residence times of between a few hours and a few days.

[00134] The present invention relates to cell based assays performed on cells confined to a microfluidic environment in contrast to cells in a micro plate well which are considered to be in a macro-fluidic environment. In a microfluidic environment, the dimensions of confinement are generally less that 500 microns wherein micro plate environments (or macrofluidic environments) the dimensions of confinement are usually> than 500 microns. For example, the linear dimensions of a 1536 well microplate are about 1.5 mm or 1,500 microns whereas a the linear dimensions microfluidic environment such as in the present invention are in the range of 50 microns to a few hundred microns.

[00135] The present invention solves several problems encountered in cell based assays in macrofluidic environments and enables new types of assays to be performed. The present invention provides solutions to the following problems that are difficult to solve in macrofluidic environments.

[00136] Small population of cells (e.g., 1-1000 cells) can be sequestered and independently assayed in a microfluidic assay region as compared to 10's of thousands of cells in macrofluidic environments.

[00137] Fluids can be delivered precisely to the cells to be assayed. Thus assay components and test compounds can be delivered at accurate concentrations and over precise time intervals.

[00138] Cells can be observed in-situ in a microfluidic device and changes in cell position or morphology can be easily determined relative to an initial starting condition, e.g., images can be taken before and after stimulation. In micro plates, for example, it is difficult to keep precise track of the location of individual cells for repeated observation whereas this is possible when cells are confined to a very specific area in a microfluidic chip.

[00139] By employing the physical properties of laminar flow and mixing by diffusion, standing concentration gradients of assay reagents can be established within an assay region of a microfluidic device according to the present invention allowing either the direct observation of biological phenomena that respond to concentration gradients or the response of a chemical biological system to the range of concentrations covered by the concentration gradient within the assay region.

[00140] In a some gradient assay preferred embodiments, the fluid velocity in the assay region can be controlled in a "closed loop" mode by providing images of the gradient, e.g., via a fluorescent tracer as feedback to the flow controller to use to adjust the fluid velocity to obtain a desired spatial characteristic of the gradient, e.g., the profile along the length and or the cross section of the assay region.

[00141] The gas permeable membrane of the present invention provides an on-chip degasser/debubbler. The gas permeable membrane allows bubbles to escape from the device both during priming and operation and it allows the gas level within the channels to be in diffusive communication and equilibrium with an external environment such as an incubation chamber or a gas manifold designed to supply test gasses to an assay region in the device. By enabling diffusive communication and rapid gas exchange between the contents of the channels, such as fluids and cells, rapid transport and equilibration of gas concentrations can be accomplished insuring adequate cellular respiration.

[00142] While impermeability to gas may not present a problem in some biochemical and cellular assays, certain other cellular assays can be enabled when gas exchange between the interior and exterior of the chip is enhanced by the use of a gas permeable material. Gas permeability ideally allows a path for gas to either enter or escape from fluids inside of microfluidics structures on the chip. The table below illustrates that a regime of operation exists for low or no flow conciliations in which the use of gas permeable materials to provide sufficient gas exchange will enhance or enable cellular assays wherein the cells are deriving the bulk of their energy from aerobic metabolism. Requirements for gas exchange to sustain optimal cellular function in a microfluidic environment depend on the flow rate of cell culture media which supports chemical nutrients and gas exchange and whether the cells are in a state of aerobic of glycolytic metabolism.

Cell metabolism	Low or no flow	High flow
Aerobic (Oxidative)	On-chip gas exchange is required to keep cells alive	On-chip gas exchange not be required if fluid is externally oxygenated
Non-Aerobic (Glycolytic)	On-chip gas exchange may not be required	On-chip gas exchange not be required if fluid is externally oxygenated

[00143] Most cells in vivo obtain their metabolic energy primarily by respiration, a process that involves the consumption of oxygen. In standard cell-culture conditions, glycolytic (non-oxygen-consuming) activity is typically increased compared to the in-vivo state, perhaps due to the somewhat hypoxic conditions that usually hold in culture [Mandel, L. (1986) "Energy metabolism of cellular activation, growth, and transformation", Curr. Topics Membr. Transport 27:261-291]. Nevertheless, aerobic metabolism continues to be vital in culture [Kemp, P., et al. (1990) "Carbohydrate and amino acid metabolism in the A10 vascular smooth muscle cell line", Biochem. Soc. Trans. 18:661; Zielke, H., et al. (1984) "Glutamine: a major energy source for cultured mammalian cells", Fed. Proc. 43:121-125].

[00144] A typical rate of oxygen consumption for a mammalian cell such as a fibroblast in culture is on the order of 10^{-16} moles O_2/s/cell [Huetter, E., et al. (2002) "Biphasic oxygen kinetics of cellular respiration and linear oxygen dependence of antimycin A inhibited oxygen consumption", Mol. Biol. Resp. 29:83-87]. If there are about 10^5

cells/cm^2 of culture surface at confluence, the oxygen consumption rate in culture is about 10^{-11} moles/s/cm^2. A microfluidic channel that is 10 μm deep and contains fluid with 200 μM dissolved oxygen contains about $2 \cdot 10^{-10}$ moles O_2/cm^2, enough to supply the needs of the cells for only about 200 s. A deep (100 μm) microfluidic channel could sustain the cells' oxidative needs for 2000 s. Both of these times are short relative to the typical times involved in culture for live-cell assays.

[00145] It can easily be shown that the presence of an oxygen-permeable membrane on the top of the microfluidic channel supplies the oxygen needs of such cells. For example, a membrane of grade 2400 Teflon AF™, oxygen permeability 990 Barrer units, 50 μm thick and subjected to an atmospheric partial-pressure difference of oxygen, passes about $1.4 \cdot 10^{-9}$ moles O_2/s/cm^2, more than a hundred times the rate required by the cells.

[00146] One of the embodiments of the present invention is a chip designed for cellular assays such as chemotaxis, cell proliferation, apoptosis, cell migration, gene and protein expression, cell to cell communication, surface and nuclear receptor binding assays, transcriptional and translational binding assays as well as any other assay that detects changes in cellular morphology or position in a microfluidic structure. The use of a gas permeable membrane and biocompatible materials allows cells to be kept alive and in certain cases to grow for extended time periods ranging from hours to weeks or months. There are certain requirements to keep cells alive and in optimal culture conditions. These include: proper temperature, proper pH, oxygen, and carbon dioxide levels, proper nutrient levels and other media factors such as growth factors, electrolytes, etc. and removal of waste products such as lactic acid and carbon dioxide. As mentioned above, if the temperature and external gas levels are kept at proper levels, such as the case in an incubator, then a chip constructed with an integrated gas permeable membrane as taught under the present invention will allow cells to thrive within the chip even in the case of low or no media flow as long as the need for nutrients and removal of waste is satisfied, for example during cell attachment or incubation. Furthermore, a chip constructed with an integrated gas permeable membrane as taught under the present invention will allow cells to survive within the chip even in the case of no media flow at room temperature and ambient conditions for short time periods (i.e.,. the time required for dispensing cells or reagents, changing media, assay read out, etc).

[00147] As mentioned previously, an additional function of the gas permeable membrane in a microfluidic device as taught by the present invention is its ability to function as an integrated degasser/debubbler. Some examples are provided which illustrate the

operation of the degassing and debubbling function. Under certain conditions, gas can be present within a microfabricated structure at a supersaturated concentration. Given the proper conditions and enough time, bubbles will form. If the concentration gradient is favorable, and the permeability of the diffusive layer is high, gas evolving from a supersaturated solution will be dissipated into the extra-chip environment rather than forming bubbles within the microstructures. Solutions can become supersaturated by any of several ways. For example, reagents stored at cool temperatures will eventually equilibrate at relatively high gas concentrations compared to the saturation level within a chip at say, 37° C. After entering the chip and reaching 37 C, bubbles will quickly form unless the gas is removed prior to the fluid entering the chip. Another example would be gas evolving from solution as a result of a chemical or electrical chemical reaction within the chip. An example of the de-bubbling function is the ability to remove bubbles introduced into a flow channel during loading or unloading fluids or when the chip is attached or removed from a fluid control fixture. Bubbles introduced into the fluid wells or flow stream will be forced out of the chip as soon as external pressure is applied.

[00148] In one embodiment, the cell cultures are frozen in a format that is ready for testing upon thawing of the cells. See, e.g., U.S. Patent No. 6,472,206 and 6,461,645, each of which is incorporated by reference in its entirety for all purposes. For example, the cells may be frozen on coverslips placed within vials (i.e., shell vials). In these embodiments, the cells are frozen on a glass substrate without the need for pre-starvation or any special handling of the cells prior to freezing. In addition, the cells do not require any special handling during thawing or use. In another embodiment, gametes from genetically engineered cells are used.

[00149] In an alternate embodiment, the cells may not be frozen but held at a lower temperature that would cause the cells to enter into a state of stasis. Chips containing frozen cells or cells in stasis can be suitably packaged so as to be archived at the factory or transported to their final users and where they can be stored and eventually thawed and used for assays without the need to load cells into the chip. Frozen chips can be used for sample archival and ultimately for screening, life science research, personalized medicine including therapy optimization, genotyping, and gene expression, and other medical diagnostics applications.

[00150] Devices of the invention are readily applicable for assays related to chemotaxis, cell proliferation, apoptosis, fluorescence polarization (ligand binding), and high content imaging. For such applications, the channel depth should be such as to accommodate

transport of cells in suspension and should be deep enough to supply adequate numbers of cells for attachment and to provide space for the formation of stable gradients in the case of gradient assays. For example, channels of about 10 microns or deeper can be used for neutrophils; about 25 microns or deeper for mammalian cells; whereas for fungi and bacterial are generally smaller in size and thus may be usable with channels less then 10 microns.

[00151] In some cellular assay embodiments, the channel depth and width are preferably optimized for the cell type to be assayed including such parameters as cell diameter when in suspension and cell height when attached in a channel as well as the density of suspended cells loaded into the channels.

[00152] In an some gradient assay embodiments, the concentration range and rate of change in concentration per unit length of the gradient is preferably selected to provide a optimal conditions for a particular assay, e.g., a specified attached cell diameter and height for a given cell type and to provide a dynamic range, i.e., minimum and maximum concentrations, and rate of change of concentration with respect to distance compatible with the distribution of cells within the assay region.

[00153] In some gradient assay embodiments, wherein a reagent having a high diffusion coefficient is used to generate a gradient, it is preferable to use a relatively higher fluid velocity to provide an assay region with a size compatible with imaging on a CCD camera.

[00154] In some gradient assay embodiments, wherein a reagent having a low diffusion coefficient is used to generate a gradient, it is preferable to use a relatively lower fluid velocity to provide an assay region with a size compatible with imaging on a CCD camera.

[00155] Figure 69 shows an exemplary 3-1 combiner structure 1060 wherein each of the three feeder channels 1062, 1064, and 1066 carries a first common reagent 1074a, 1074b, and 1074k, respectively. It should be noted that the structure is not limited to three feeder channels. The three feeder channels 1062, 1064, and 1066 then merge into a single main channel 1068. Dotted lines 1070 indicate the boundaries between the laminar flow streams after the merger which do not mix other than by diffusion. The concentration of common reagent 1074 is constant across the channel and is independent of position in the channel. For typical flow velocities and channel dimensions (in the range of 1 microns to 1000 microns in width and depth), Reynolds numbers are low and flow patterns are laminar. In laminar flow, by definition, there is no turbulence and mixing is governed by diffusion which occurs during the transit time in the channel. The rate of diffusion is determined by the

diffusion coefficient of the diffusing species in the solute, typically water in most biological applications. The diffusion coefficient is linearly dependent on the radius of the diffusing species (i.e., the size of the molecule or particle) and the inversely dependent on the viscosity of the solute.

[00156] Figure 70 shows the structure 1080 of figure 69, wherein each of three channels 1062, 1064, and 1066 carries a common first reagent 1074. The three feeder channels 1062, 1064, and 1066 then merge into a single main channel 1068. Dotted lines 1070 indicate the boundaries between the laminar flow streams after the merger which do not mix other than by diffusion. A second reagent 1082 is added to the outer channels causing a standing concentration gradient to form in the main channel 1068 as second reagent 1082 diffuses across flow boundary lines 1070 towards the center of the channel.

[00157] Figure 71 shows a plot 1090 of the concentrations of first reagent 1062 and second reagent 1082 as a function of distance across the channel in structure 1080 of figure 70 at an upstream location in the main channel 1072a relative to the merge point of channels 1062, 1064, and 1066.

[00158] Figure 72 shows a plot 1100 of the concentration of first reagent 1062 and second reagent 1084 in structure 1080 of figure 70 at a downstream location in the main channel 1072k relative to the merge point of channels 1062, 1064, and 1066. Since mixing occurs only by diffusion across flow boundaries 1070, concentration profile 1094 of second reagent 1082 exhibits a steep gradient at upstream location 1072a whereas at location 1072k second reagent 1082 has diffused across flow boundaries 1070 and into the central region of channel 1068 exhibiting a shallower concentration gradient 1094. The extent to which second reagent 1082 diffuses into the central region depends on a balance between the flow rate, the channel width, and the diffusion coefficient of the second reagent. For example, if the second reagent is a large protein, it will diffuse slowly and the gradient will remain steep until many seconds of transit have passed (.i.e., a long channel and or low flow velocity will be required to provide the time required for significant diffusion to take place). It is useful to note that diffusion rate depends linearly on the molecule size and the flow rate but has a square law dependence on the channel width. Diffusion coefficients range from 10E-5 cm^2/second for small molecules to 10E-7 cm^2/second for large bio-molecules to 10E-10 cm^2/second for cells. Computer simulations of diffusion under representative operating conditions and geometries have been carried out and have been found to correlate well with results obtained with actual gradients observed using fluorescein as a fluorescent tracer in a fabricated device. Images of the gradients in channels were recorded with a CCD camera

through a fluorescence microscope and were found to correlate well with the simulation results.

[00159] Figure 73 shows a plan view 1110 of a 3-1 combiner structure similar to that of figure 69 wherein each of the three channels 1062, 1064, and 1066 carries a common reagent 1074 and cells 1112 have been loaded into assay region 1114 in main channel 1068. In this embodiment, cells 1112 (e.g., neutrophils) are introduced into main channel 1068 and allowed to attach to the channel walls. Drawing on the principles illustrated in the previous figure, after the introduction of second reagent, e.g., a chemo-attractant, into outer channels 1074a and 1074k, a gradient in concentration the chemo-attractant will form between the outer regions 1118a and the central region 1116 of channel 1068 downstream from the junction of channels 1074a, 1074b, and 1074k, and along the channel between locations 1072a and 1072k as illustrated in figures 71 and 72. The gradient from the center 1116 to the edge 1118a of the channel decreases in slope as the downstream distance from the junction increases. The precise shape of the concentration gradient and location of along the channel of point depends on the diffusion coefficient of the chemo-attractant and the flow rate, and the channel width.

[00160] Figure 74 shows a plan view 1130 of the 3-1 combiner structure of figure 73 wherein a second reagent, i.e., a chemoattractant, has been added to the outer channels causing the cells to migrate in response to the concentration gradient of the second reagent from outer regions 1118a to center region 1116 formed along main channel 1068. Cells 1112 that respond to chemo-tactic agents such as neutrophils migrate in the direction of the increasing chemo-attractant concentration. Images of the relative cell positions taken at intervals after stimulation with the chemo-attractant can provide a kinetic readout of cell migration which is difficult to obtain with the methods of the prior art. Images can be compared with one an other using computer algorithms to quantitative the changes in physical position. For example, autocorrelation algorithms can be used to provide a quantitative indication of the extent to which cells have moved from the initial positions even if the movement is small. Also, since the concentration gradient decreases in steepness with increasing distance from the 3-1 junction, this assay provides quantitative information on the relationship of cell migration characteristics to the magnitude of the concentration gradient. Since in assay region 1112 of channel 1068 cells can be individually imaged and observed, in a preferred embodiment, the responses of cells could be quantified and graded to provide additional information on the behavior of individuals within a population of cells. Specific reagents, such as stains, protein and nucleic acid binding probes, and the like could be added

to detect certain properties of cells such as expression (or lack thereof) of specific proteins or signaling molecules in order to sub-type a population of cells subjected to a particular reagent protocol For example, CHO (Chinese Hamster Ovary cells) were loaded into in a chip fabricated according to the present invention comprising a 3-1combiner similar to structure 1110 of figure 73. A chip containing the cells was incubated for 24 hours at 37 ° C and then removed from the incubator and subjected to a Trypan Blue uptake assay. The cells in the 3-1 combiner were deemed to have passed the Trypan Blue uptake assay, i.e., (negligible update of Trypan Blue).

[00161] In a gradient assay embodiment, a uniform "lawn" or monolayer of cells 1112 is preferred attached within channel 1068 and a uniform, unperturbed gradient is formed over the uniform lawn of cells within assay region 1114.

[00162] In an alternate gradient assay embodiment, the cells are deposited and attached within channel 1068 either in a sparse monolayer or in small clumps within assay region 1114 and preferably, the attached height of the cells or small clumps of cells is less than about one half of the channel depth so as to provide enough overhead space within the channel for a standing gradient to be minimally perturbed by the presence of the cells.

[00163] Figure 75 illustrates an exemplary method for loading cells 1112 into main channel 1066 from the center channel 1064 of the 3-1 combiner structure 1160. In this embodiment, cells (e.g., neutrophils) are introduced from center channel 1064. It is assumed that all channels terminate at access ports to which appropriate pressures are applied to cause the desired flows to occur. Because the diffusion coefficient of cells is very small compared to the diffusion coefficient of most anticipated chemo-attractant molecules, the cells will be expected to remain in their flow stream for extended time periods regardless of whether or not the cells are flowing in the stream or stopped. If the flow is stopped, gravity will cause the cells settle to the bottom of the channel where they will attach to the channel floor. The unassisted settling time should be only a few seconds if the cells are denser than the medium as is common in practice.

[00164] Figure 76 shows a plan view 1170 of the 3-1 combiner structure of figure 75 wherein a second reagent has been added to the outer channels 1062 and 1066. In analogous fashion to the embodiment in figures 74, after the chemo-attractant is introduced to channel 1066 in structure 1170, a concentration gradient of the chemo-attractant will form between the outer regions 1118b and the central region 1116 of the channel downstream of the 3-1 junction at locations 1072a through 1072k along the channel. The gradient from the center region 1116 to outer regions 1188 decreases in slope as the distance from the 3-1 junction

increases. The precise shape of the concentration gradient will depend on the diffusion coefficient of the chemo-attractant and the flow rate, and the channel width. Cells that respond to chemo-tactic agents such as neutrophils will then migrate in the direction of the increasing chemo-attractant concentration. Images of the relative cell positions taken at intervals after stimulation with the chemo-attractant will provide a kinetic read out of the migration of the cells. Images can be compared with one an other using computer algorithms to quantitative the changes in physical position. For example, autocorrelation algorithms can be used to provide a quantitative indication of the extent to which cells have moved from the initial positions even if the movement is small. Also, since the concentration gradient will decrease in steepness with increasing distance from the 3-1 junction this assay will also provide quantitative information on the relationship of the rate of cell migration to the magnitude of the concentration gradient.

[00165] Figure 77 illustrates an exemplary method for loading cells 1189 into the assay region of H structure 1180 from one of the side branch channels 1182. In this embodiment, cells 1190 (e.g., neutrophils) are introduced into channel 1182. A first reagent e.g., culture media is introduced into both channel 1182 and 1186 and therefore the concentration of the first reagent is flat across the channel in the assay region 1195. It is assumed that all channels terminate at access ports to which appropriate pressures are applied to cause the desired flows to occur.

[00166] Because the diffusion coefficient of cells is very small compared to the diffusion coefficient of most anticipated chemo-attractant molecules, the cells will remain in their flow stream for many hours regardless of whether or not the cells are flowing in the stream or stopped. If the flow is stopped, gravity will cause the cells settle to the bottom of the channel where they will attach to the channel floor within a few minutes to an hour.

[00167] Figure 78 shows the 3-1 combiner structure 1196 of figure 77 wherein a second reagent has been added to channel 1186. After introduction of a second reagent, e.g., chemo-attractant, a steeply sloped chemical gradient of the chemo-attractant 1198 will form in the central region of the channel slightly downstream from the "T" junction formed by the intersection of channels 1186 and 1182. The gradient decreases in slope as the distance away from the T junction increases to resemble the shallow profile 1199 near the downstream T junction. The precise shape of the concentration gradient in assay region 1195 along the central bridging channel depends on the diffusion coefficient of the chemo-attractant, the fluid flow rate, the channel width and depth. Cells 1190 responding to a concentration gradient of a chemoattractant migrate within assay region 1195 from starting position 1189 to

a new position 1197 in the direction of the increasing chemo-attractant concentration after the passage of time. Images of the relative cell positions taken at intervals after stimulation with the chemo-attractant will provide a kinetic read out of the migration of the cells. Images can be compared with one an other using computer algorithms to quantitative the changes in physical position. For example, autocorrelation algorithms can be used to provide a quantitative indication of the extent to which cells have moved from the initial positions even if the movement is small. Also, since the concentration gradient will decrease in steepness with increasing distance from the 3-1 junction this assay will also provide quantitative information on the relationship of the rate of cell migration to the magnitude of the concentration gradient.

[00168] Figure 79 shows a plan view 1200 of an exemplary embodiment of a perfusion chamber 1206b with a shape designed to efficiently perfuse and / or purge assay region 1208a in a microfluidic perfusion chamber 1204a comprised of inlet port 1202a, channel section 1206a, perfusion chamber 1206b, channel section 1206c, and outlet port 1202b. A key feature of perfusion chamber section 1206b is that the walls of the chamber are shaped to be parallel to equipotential lines in the fluid flow field thus minimizing the fluidic resistance of the chamber and the time to purge the chamber when switching from one reagent to another.

[00169] In an alternative preferred embodiment, mechanically activated valves would be positioned over the mid points of channel sections 1206a and 1206b of perfusion structure 1200 by suitable mounting and positioning of external mechanical actuators similar to actuator 234 of figures 9-11. With the valves in the open position, fluid is flowed through the perfusion chamber carrying with it a chosen reagent such as cells, beads, particles, bio-molecules, chemicals and the like until the chamber if purged and filled with fluid. Both valves are then closed and the fluid trapped between the valves the chamber 1208a is allowed to evaporate through the membrane taking advantage of the fact that gas permeable membranes such as those used preferably to build chips according to the present invention are also permeable to water vapor and other volatile dissolved components but not permeable to large molecules, cells, beads, and the like. As the fluid evaporates through the membranes with the channel sections 1206a and 1206b, the membrane collapses trapping the contents of the channel between the membrane and the channel surface.

[00170] There are numerous potential uses for the embodiment evaporative trapping device described above including but not limited to the archival of cells and bio-molecules as well as the creation of handling and storage devices and systems for nanoparticles, sensor molecules, fluorescent and absorbent dyes and the like. Further by combining the inherent

accuracy and precision of the processes used to fabricate the channels with the ability to trap small sized substances therein, it is possible to create specific shaped chambers for trapping arrays of beads, cells or other substances within the chamber for use as physical, spectroscopic, and chemical, and biochemical reference and calibration standards. If the concentration of the substance to be trapped is known, then the number of trapped items in the chamber can be calculated by multiplying the volume of the chamber by the concentration of the items to be trapped giving the number of items to be trapped. If the concentration is precisely known as is the case with standards such as beads or cells that have been counted, it is possible to predict the number of particles that will be trapped within the chamber.

[00171] Figure 80 shows a plan view 1210 of an exemplary dead-end channel 1206f between channel sections 1206d and 1206e along main channel 1204b extending between access port 1202a and 1202b. Dead-end channel 1206f is inefficiently purged by the flow in the main channel and therefore gas is trapped within dead-end channel 1206f as shown in figure 80 unless pressure is applied to both access ports 1202a and 1202b and the gas in dead-end channel 1206f is forced to exit through a gas permeable membrane above dead-end channel 1206f, e.g., (FIG 6).

[00172] A preferred embodiment of structure 1210 provides a method for loading cells or other reagents into one of the access ports and trapping the cells or other reagent in dead-end channel section 1206f. In a preferred method, cells are first flowed through channel 1204b from access port 1202a to 1202b thus filling channel section 1204b with cells. Next, pressure in the range of 2-10psi is applied to both access ports 1202a and 1202b causing dead-end channel section 1206f to fill with fluid containing cells and / or other reagents. When normal flow is reestablished in channel 1204b, by applying pressure between access ports 1202a and 1202b, dead-end channel 1206f is inefficiently purged, or bypassed by flow through channel section 1204b allowing the trapped cells or other reagents to reside in dead end channel 1206f. The relatively low purging efficiency of dead-end channel 1206f having a long and thin shape can be understood intuitively by comparison to the high relative purging efficiency of perfusion chamber 1200 having a streamlined oval shape.

[00173] In an alternate preferred embodiment, a small hole or potentially a plurality of holes are fabricated near the end of channel section 1206f opposite main channel section 1204b to allow gas to escape at a higher rate than would occur by gas permeation through the membrane alone. In some assays, with cells for example, it may be desirable to fill the dead-end quickly relatively to the settling rate of the cells. The technique of using of holes with a diameter small enough to allow gas but not fluid to escape is well known by those skilled in

the art. The diameter chosen for the hole should be large enough to allow sufficient gas to escape to achieve the desired filling rate and small enough to prevent fluid flow by maintaining the surface tension barrier provided by a small hole in the hydrophilic membrane. Holes in the range of a few microns in diameter up to tens of microns in diameter can be economically fabricated with laser or photolithography based tools. Holes of tens of microns and larger can be fabricated my mechanical tooling methods.

[00174] This method can be used to trap cells for use in assays including but not limited to cell migration assays wherein, after the cells are trapped and attach to the surface of channel 1206f, a concentration gradient of a chemoattractant is established near the intersection of dead-end channel 1206f and channel section 1204b in the presence of cells trapped in the dead-end channel. Chemoattractant is preferably dispensed into one of the access ports and flowed through channel section 1204b by the application of a pressure differential between the two access ports. This method of trapping cells in a dead-end channel can also be sued to other substances including but not limited to non-adherent cells, beads, particles, fluorescent dyes, biomolecules of all kinds for use in various applications and assays in a manner distinctly different from the use of size filters or sieve methods used in the prior art.

[00175] Figure 81 shows a plan view 1220 of an exemplary standard unit cell with an H structure configuration having a valve 1222 located at the center region of the H bridge channel. With valve 1222 in the closed position, the structure is equivalent to two of the structures shown in figure 80 since 1204c can be seen to have a first dead-end channel intersecting a main channel extending between ports 1 and 2 and a second dead-end channel intersecting a main channel extending between ports 3 and 4. This structure can be used to perform assays in ways similar to the embodiments of figures 64 through 67 where cells, beads, or other particles are first flowed through the H structure and then valve 1222 is closed creating two dead-end channels which entrap the contents of the fluid. It is useful to point out that the diffusion coefficient of cells and large molecules and beads is very small forcing these particles to remain virtually suspended in position affected primarily by gravitational forces and shear forces of fluid flowing in the main channel.

[00176] Figure 82 shows a plan view 1230 of an exemplary method of loading cells 1231into an H structure from a side branch 1232, the H structure having a valve 1235 in a central region to trap the cells. Those skilled in the art will recognize that beads, particles, nanoparticles, or other substances could readily be substituted for the cells used in this example. Cells 1231 are flowed through feeder channel 1232 with the flow being split

equally between channels 1234 and the bridge of the H structure past valve 1235 the flow then splitting again between exit channels 1236 and 1238. It is assumed that all channels terminate at access ports to which appropriate pressures are applied to cause the desired flows to occur.

[00177] Figure 83 shows a plan view 1240 of the H structure of figure 82 with valve 1235 closed and the cells1231 trapped within assay regions 1240a and 1240b of the two dead-end channels created by the closed valve.

[00178] Figure 84 shows a plan view 1250 of the structure of figure 83 after continued flow has washed away the not trapped in the assay regions 1242b of the dead-end channels.

[00179] Figure 85 shows a plan view 1260 of structure of figure 84 after performing an assay, wherein a second reagent is added, e.g., a chemoattractant which causes the trapped cells to migrate from their initial positions within assay regions 1242a and 1242b into new positions extending further into the main channel by following the chemoattractant concentration gradient extending between the dead-end channel and the main channel. In a first example of an alternate preferred embodiment, after re-opening valve 1235, the cells could be replenished and the assay repeated. In second alternate preferred embodiment, a sequence of reagents could be flowed over the cells after they became adhered within the dead-end channel. In a third alternate preferred embodiment, using non-adherent cells and a dead end channel designed with the proper shape aspect ratio to accommodate the desired cell type, it is possible to dose the non-adherent cells trapped in the dead-end channel with test reagents by flowing test reagents in the main channel past the junction of the dead-end channel. Those skilled in the art will recognize that this structure could be used to implement many other functions which could be used in various types of chemical, biochemical, and cellular assays.

[00180] Figure 86 shows an exemplary embodiment 1270 of a two compartment device wherein cell type A 1288 are loaded into a first compartment 1272 through a first channel 1280. A slotted sieve structure 1276 separates a first compartment 1272 and a second compartment 1274. It is assumed that all channels terminate at access ports to which appropriate pressures are applied to cause the desired flows to occur. A first cell type A 1288 is introduced into compartment 1272 by way of flow through first channel 1280 and third channel 1284. The sieve is fabricated by etching slots 1278 into the top of the wall between first compartments 1272 and second compartment 1274. The depth of the slots is chosen to be slightly smaller than the nominal diameter of cell type A.

[00181] Figure 87 shows a plan view 1290 of the two compartment device of figure 86

after the introduction of a reagent that induces cell migration from the first compartment 1272 into the second compartment 1278. Once the cells are trapped, and stabilized in first compartment 1272, chemo-attractant is introduced by flow through first channel 1284 and fourth channel 1286. The introduction of the chemo-attractant through channel 1284 (or alternately channel 1280) causes a concentration gradient between regions of first compartment 1272 and second compartment 1274 and initiates the migration of cells under the influence of the concentration gradient between the two chambers. Note that the cells assume an elongated shape 1289 as they squeeze through the physical restriction in the sieve. This is similar to their actual environment wherein they elongate and pass through tissue on their way to their final destination. Concentration gradients can be controlled by the magnitude of the flow rates in channels 1280, 1282, 1284, and 1286. As in the previous embodiment, images of the relative cell positions observed at intervals after stimulation with the chemo-attractant will provide a kinetic read out of the migration of the cells. Images can be compared with one an other using computer algorithms to quantitative the changes in physical position. For example, autocorrelation algorithms can be used to provide a quantitative indication of the extent to which cells have moved from the initial positions even if the movement is small.

[00182] In an exemplary alternate preferred embodiment, a second cell type B is introduced into second compartment 1274 by way of channels 1282 and 1286. Once the cells are trapped, and stabilized, a chemical stimulant is then introduced by flow through channel 1284 and channel 1286. The introduction of the stimulant causes a concentration gradient between first compartment 1272 and second compartment 1274 whereas introduction of stimulant through channels 1284 and 1280 in first compartment 1272 and / or channels 1282 and 1286 in second compartment 1274 allow the possibility of stimulating the cells in first compartment 1272, second compartment 1274 or both compartments . Concentration gradients can be controlled by the magnitude of the flow rates in channels 1280, 1282, 1284, and 1286. In this embodiment, it is possible to study the interactions of more than one cell type and the effects of more than one substance on the two cell types individually or in combination. As in the previous embodiment, images of the relative cell positions or morphologies taken at intervals after stimulation with the chemo-attractant will provide a kinetic read out of the migration or morphological changes of the cells. Fluorescence images can also be obtained when it is desired to read out a fluorescent signal. Other types of detection and imaging strategies can be envisioned to work with this cell to cell communication chamber. Images can be compared with one an other using computer

algorithms to quantitative the changes in morphology, physical position, or fluorescence. For example, autocorrelation algorithms can be used to provide a quantitative indication of the extent to which cells have changed shape or moved from the initial positions even if the movement or shape change is small. Similarly changes in fluorescence intensity, fluorescence resonance energy transfer, fluorescence lifetime, fluorescence polarization, or fluctuation correlation spectroscopy can also be used to detect changes in the cells due to stimulant addition or other assay protocols.

[00183] Figure 88 provides an illustrative example 1300 of bell shaped 1306 and saturating 1308 dose-response curves. Dose-response curves provide a fundamental read out for many important biological functions such as response to a chemoattractant is in curve 1306 or a receptor-ligand binding curve as in curve 1308. Using the methods taught by the present invention wherein standing concentration gradients are induced within microfluidic channels or chambers, it is possible to construct assays that provide dose-response information as a way of reading out assay information.

[00184] Dose-response assays within a microfluidic environment using standing concentration gradients inherently eliminates pipetting errors and well to well variations since errors in pipetting cause offsets which can be calibrated and removed by techniques like ratiometric correction. Dose-response information obtained from a localized population of cells, for example, has the potential to reduce or eliminate errors caused by curve fitting and averaging of multiple data points as has been done in the prior art. Additionally, obtaining assay responses in terms of smooth data curves allows the observation of subtle fluctuations and inflection points which potentially may reveal the mechanisms of action of the underlying biological systems. This information could be quite valuable in pharmacology for determining the mechanisms of action of both therapeutic drugs as well as toxic substances. In current practice, dose-response curves are used to validate assays and for pharmacology studies. Typically, 10-100 individual measurements are required so that each point on a dose-response curve consists of the average of multiple individual measurements. Many times, the inflection point of the dose response curve contains the most valuable information and the accuracy with which the inflection point can be determined is limited by the number of different concentration points assayed, by pipetting errors and other well to well errors associated with microplates and readers thereof.

[00185] The present invention provides microfluidic perfusion chambers into which relatively small numbers of cells can be loaded and subjected to stable, continuous concentration gradients. Specific structures can be constructed to break up large

concentration ranges required for certain dose-response curves into several smaller ones designed to cover regions of specific such as inflection points and regions such as saturation or desensitization such as in the dose-response curves 1306 and 1308 shown in figure 88.

[00186] An additional benefit of dose-response or variable gradient assays according to the present invention is the ability to accommodate primary cells from different individuals and expected the expected day to day variations in their responses. For example, bell shaped dose-response curve 1306 is typical of the response of a motile cell to a chemoattractant. To meaningfully assay these cells, it is often necessary to carry out the assay at the peak response point. In a gradient assay according to the present invention, a continuum of data points are collected over a range will dramatically increases the probability the peak response will be captured. Those skilled in the art will recognize that a continuous dose-response assay in a microfluidic environment can provide many benefits and much information that is difficult or impossible to collect using methods in the prior art and are not limited to the few illustrative examples used herein to illustrate the potential uses of this powerful technique.

[00187] Figure 89 shows a top view of 3-1 combiner structure 1320. A first reagent 1330 flowing in channel 1322 and a second reagent 1334 flowing in channel 1326 combine with a third fluid 1332 flowing in center channel 1324 after entering main channel 1328 and exit the main channel at 1336. Overlapping standing concentration gradients 1340 and 1342 form as first reagent 1330 and second reagent 1334 mix by diffusively crossing fluid stream 1338 which is present due to the injection of fluid 1332 from channel 1324 into main channel 1328.

[00188] Computer simulations of diffusion under representative operating conditions and geometries have been carried out and have been found to correlate well with results obtained with actual gradients observed using fluorescein as a fluorescent tracer in a fabricated device. Images of the gradients in channels were recorded with a CCD camera through a fluorescence microscope and were found to correlate well with the simulation results. In a preferred embodiment, overlapping standing gradients can be used to study the effects of many different experimental conditions at once.

[00189] With a two dimensional array of sensing elements covering the floor of channel 1328, it is possible to measure the change in a property of a sensing element at various positions within the main channel 1328 of 3-1 combiner 1320 and thus simultaneously perform a number of experiments limited by the number of discrete sensing elements and the smallest resolvable change in reagent concentrations. In a preferred embodiment with cells as the sensing element and the first and second reagents were two

drugs known to stimulate the cells in a measurable way, the effects of the two drugs in various combinations could be studied.

[00190] In an alternative preferred embodiment, a third reagent 1332 could be added through channel 1324 such that three overlapping concentration gradients would be present within channel 1328. Region 1346 represents a region where all three regents would be present. The number of channels in such a combiner structure could be increased or decreased and the design otherwise changed to suit the needs of a particular experiment of product. Using methods to trap and attach cells as taught by the present invention combiner 1320 can be used to implement dose-response assays wherein each individual cell can be thought of as an individual sensing element. Alternatively, a lawn of cells, beads, sensor molecules, nanoparticles, or other self assembling structures placed within channel 1328 in the presence of single or multiple overlapping gradients could perform a two dimensional or multi-parameter readout function enabling many physical, chemical, biophysical, or biological experiments to be carried out simultaneously.

[00191] Those skilled in the art could readily envision other ways to make use of this method for performing gradient assays and experiments where the effects of two or more test substances are simultaneously evaluated.

[00192] Figure 90 illustrates an exemplary method wherein multiple cell types, bead types, or other sensor element types are loaded into the main channel 1358 of a 3-1 combiner structure 1350 so as to carry out various types of multi-parameter assays. The 3-1 combiner 1350 is operated in a manner similar to that of figure 89 and similar standing concentration gradients are established in main channel 1376 by the injection of various combinations of reagents 1360, 1362, and 1364 into channels 1152, 1354, and 1356, respectively. For the exemplary multi-cell assay shown, a first cell type 1374 is injected into the center region of the main channel 1358 from the center channel 1354. Flow is stopped and the cells are allowed to attach to the channel wall. A second cell type 1368 is injected into the left section of the main channel from left channel 1352. Flow is stopped and the cells are allowed to attach to the channel wall. After the first and second cell types have stabilized, a first reagent 1364 can be introduced from the right channel 1356 whereupon after entering main channel 1358 reagent 1364 diffuses into the center portion of the channel containing first cell type 1374. A standing gradient of the concentration of first reagent will be set up along the channel over the first and second cell types as the first reagent diffuses toward the edge of the channel as flow proceeds down the channel indicated by arrow 1376. Cells 1374 under stimulation by the first reagent may be induced to secrete a compound that could act as a

second reagent either a stimulant or potentially a toxin to second cell type 1368 on the left side of the main channel 1358. Assays of this type could preferably be used to study the interactions of primary and cultured cell types for organ system and tissue interface models. The compounds secreted by first cell type in response to first reagent could be the target compound to be studied or it could be desired to study the effects of a drug in which the target compound would be the first reagent, for example a drug candidate.

[00193] One skilled in the art will recognize that there are many possible uses for the 3-1 combiner as well as the other microfluidic structures described herein and that many configurations of channels, chambers, valves, and the like are possible to implement various kinds of important chemical, biochemical, and biological assays and protocols for carrying out these assays according to the methods as taught by the present invention. Many types of chemical, biochemical, and cellular assays including but not limited to cell migration, cell motility, cell-cell communication, cellular viability, cellular toxicity, cellular proliferation, gene and protein expression, receptor, enzyme, nucleic acid and protein binding, receptor, as well as enzyme, nucleic acid, and protein functional, assays could be designed to take advantage of the methods taught according to the present invention. Additionally, the structures and methods taught by the present invention could be readily adapted by those skilled in the art for use in the development of organ system and tissue interface models including but not limited to gut, liver, epithelia, endothelia, kidney, and brain.

[00194] All patents and publications mentioned in the specification are indicative of the level of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference, including but not limited to the following, to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

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1. Owicki et al, "Biosensors based on the energy metabolism of living cells: The physical and chemistry and cell biology of extracellular acidification," Biosensors and Bioelectronics, 7, pp 255-272 (1992).

2. Gravesen et al, "Microfluidics – a review," J. Micromech. Microeng., 3, pp 168-182, (1993) UK

3. Jeon et al., Neutrophil chemotaxis in linear and complex gradients of interlukin-8 formed in a microfabricated device, "Nature Biotechnology, V 20, July 2002.

What is claimed is:

1. A microfluidic device comprising:

a fabricated substrate having at least one inlet access port disposed in said substrate;

at least one channel disposed in said substrate and connected to said inlet access port; and

a gas permeable membrane sealably attached to said substrate to cover said channel.

2. The device of claim 1 further comprising one or more of the following:

at least one outlet access port disposed in said substrate and connected to said channel;

said channel is less that 500 microns in width and depth;

one or more fluid chambers disposed in said substrate and connected to said channel;

means for delivering one or more gases to the surface of said gas permeable membrane; and

means for controlling the velocity of a fluid in the channel.

3. The device of claim 2 wherein said substrate is a microscope slide or a microplate.

4. The device of claim 1, further comprising a surface coating on the channel and/or membrane.

5. The device of claim 1 further comprising one or more cells.

6. The device of claim 1 further comprising one or more reagents wherein at least one of said reagents is present in a concentration gradient.

7. The device of claim 1 further comprising one or more beads or particles.

8. The device of claim 1 wherein a portion of the membrane can be deflected into or away from said channel or said substrate.

9. The device of claim 8, wherein said membrane can be deflected by application of a mechanical force, pneumatic pressure, or hydraulic pressure.

10. An array comprising one or more positionally distinguishably devices of claim 1.

11. The array of claim 10 comprising a plurality of devices of claim 1 and further comprising a interconnecting channel network connecting said devices.

12. A method of performing an assay to evaluate a property of a compound comprising the steps of:

providing a device of claim 1;

introducing said compound into said device; and

evaluating said property of said compound.

13. The method of claim 12 wherein said device further comprises primary or immortalized eukaryotic or prokaryotic cells.

14. The method of claim 13 wherein said property is said compound's effect on chemotaxis, cell proliferation, apoptosis, cell migration, cell-to-cell communication, gene expression, protein expression, or receptor binding.

15. The method of claim 13 wherein either before or after said compound is introduced into said device, said method further comprises providing introducing a reagent into said device such that said reagent is disposed in a concentration gradient in said device.

16. A method for preparing a microfluidic device having an integrated gas permeable membrane comprising the steps of:

providing a substrate having at least one channel and at least one inlet access port wherein said inlet access port is connected to said channel;

attaching a gas permeable membrane to said substrate to cover said channel;

providng a package having at least one fluid well wherein said fluid well corresponds to said inlet access port; and

contacting said package with said substrate to form a microfluidic device.

17. The method of claim 16 wherein said membrane comprises polymethylpentene.

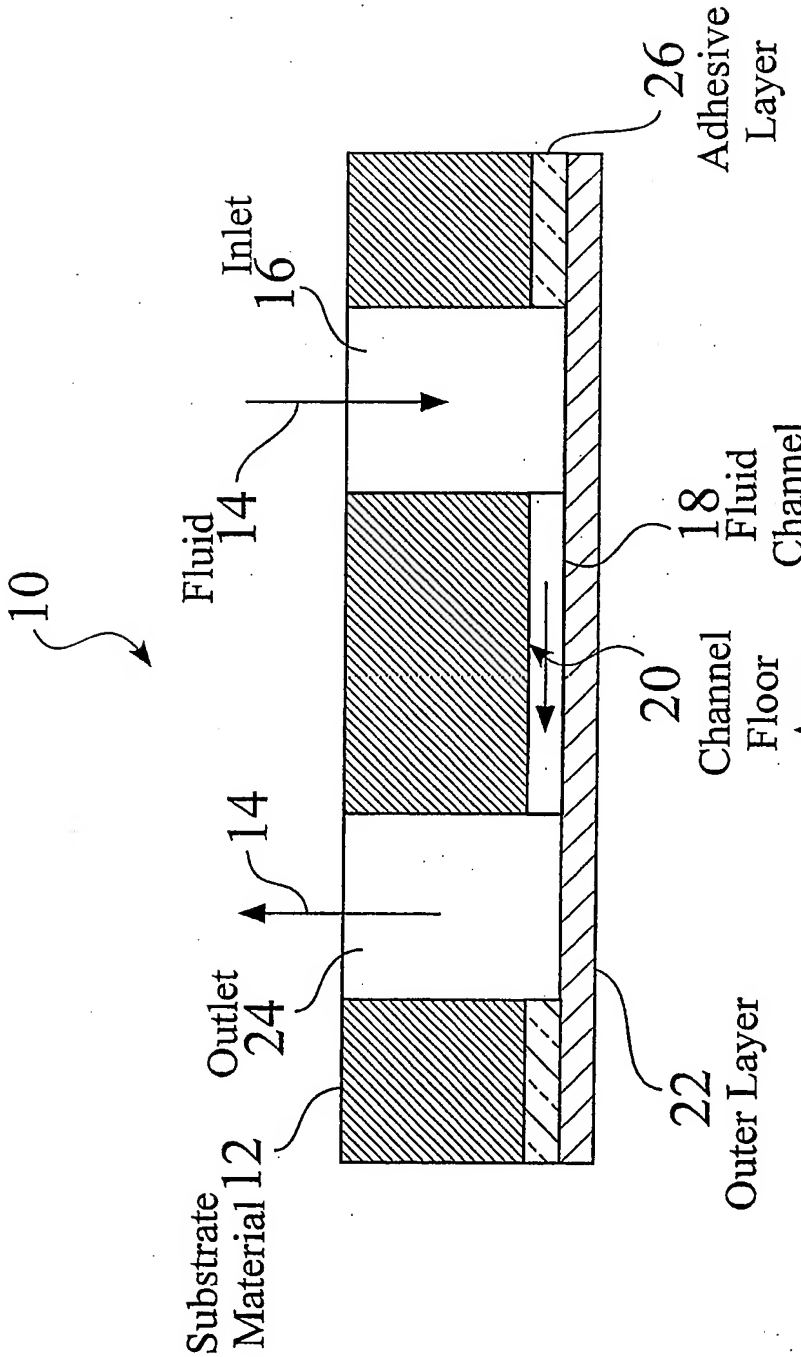


Fig. 1
PRIOR ART

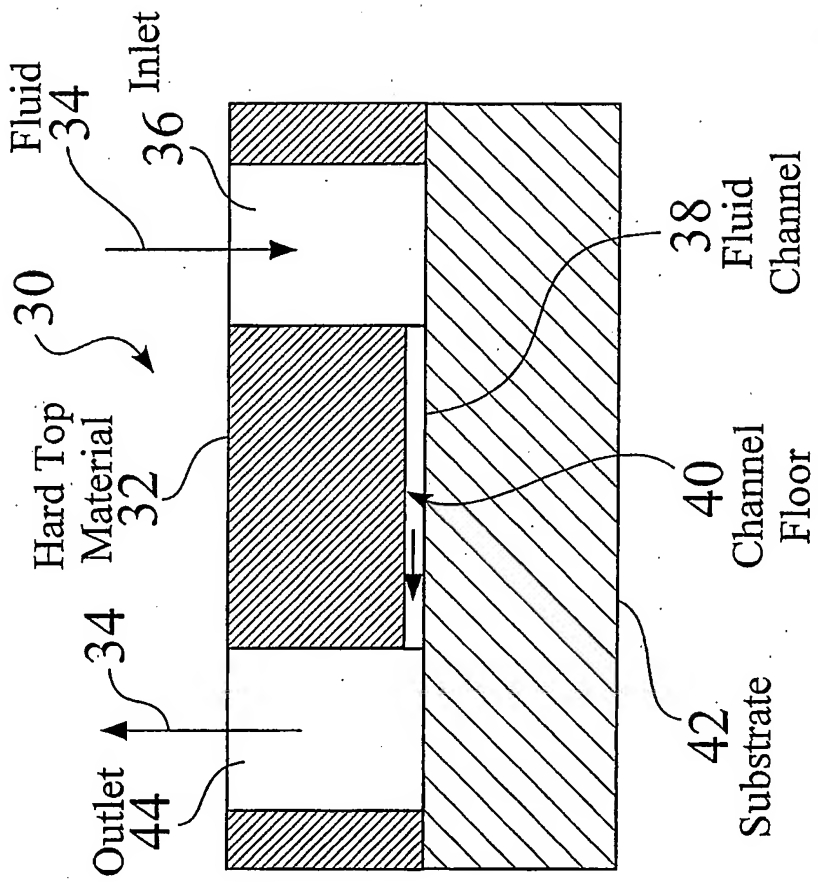


Fig. 2
PRIOR ART

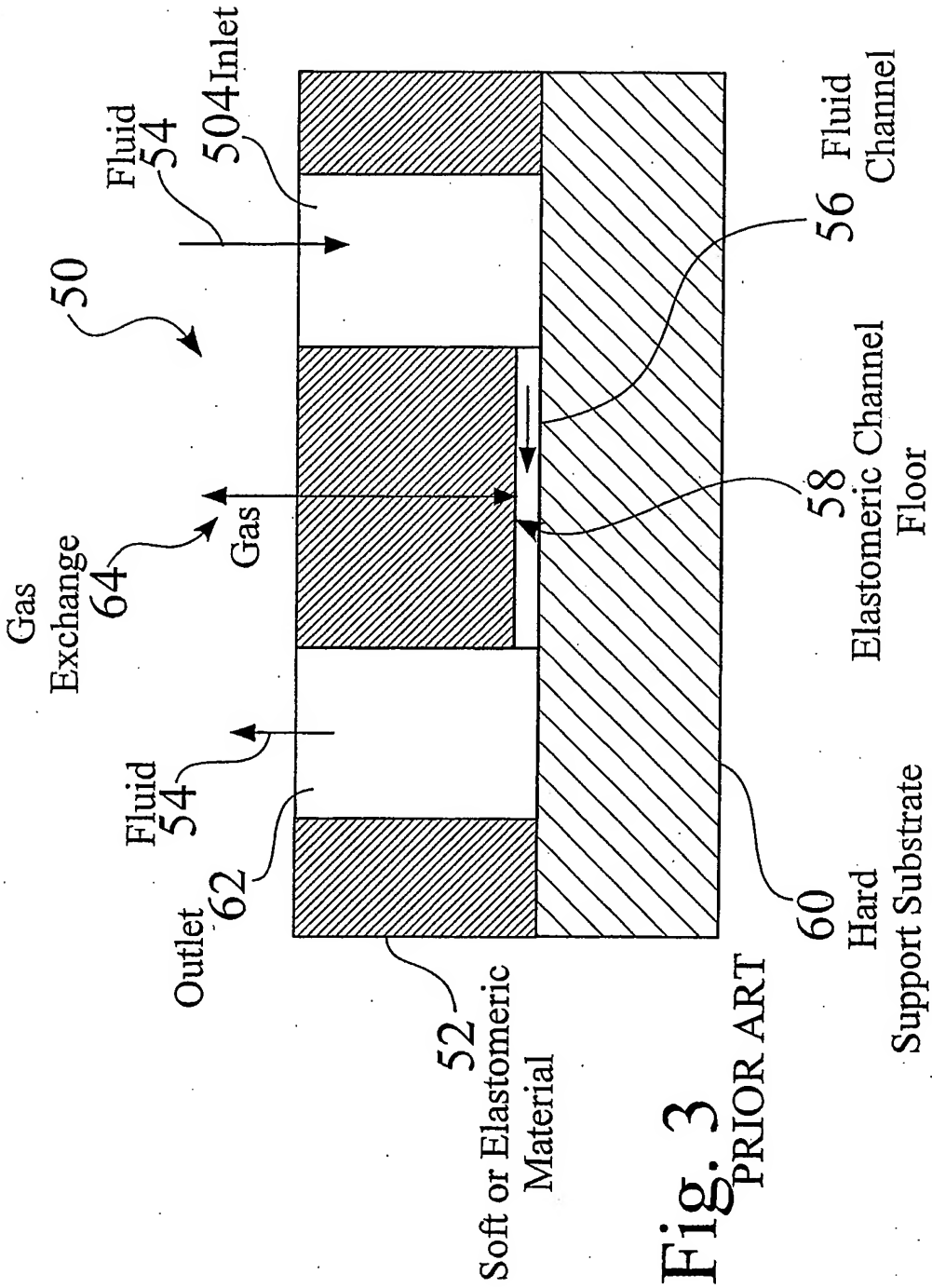


Fig. 3
PRIOR ART

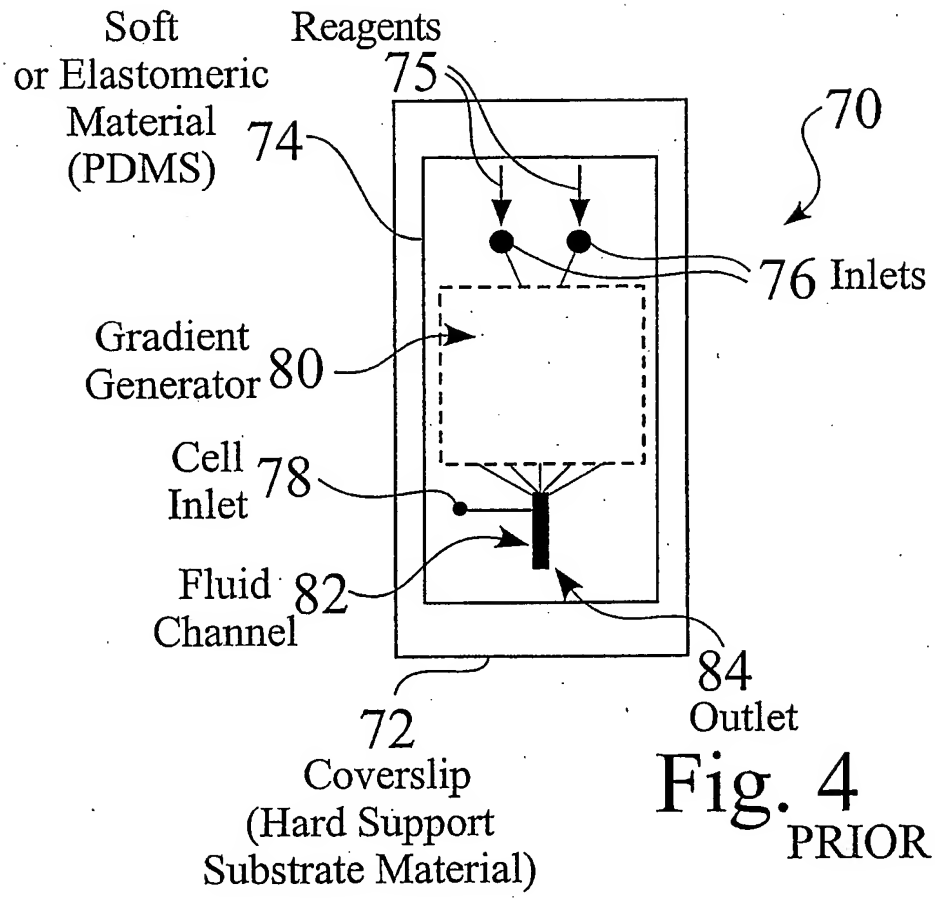


Fig. 4
PRIOR ART

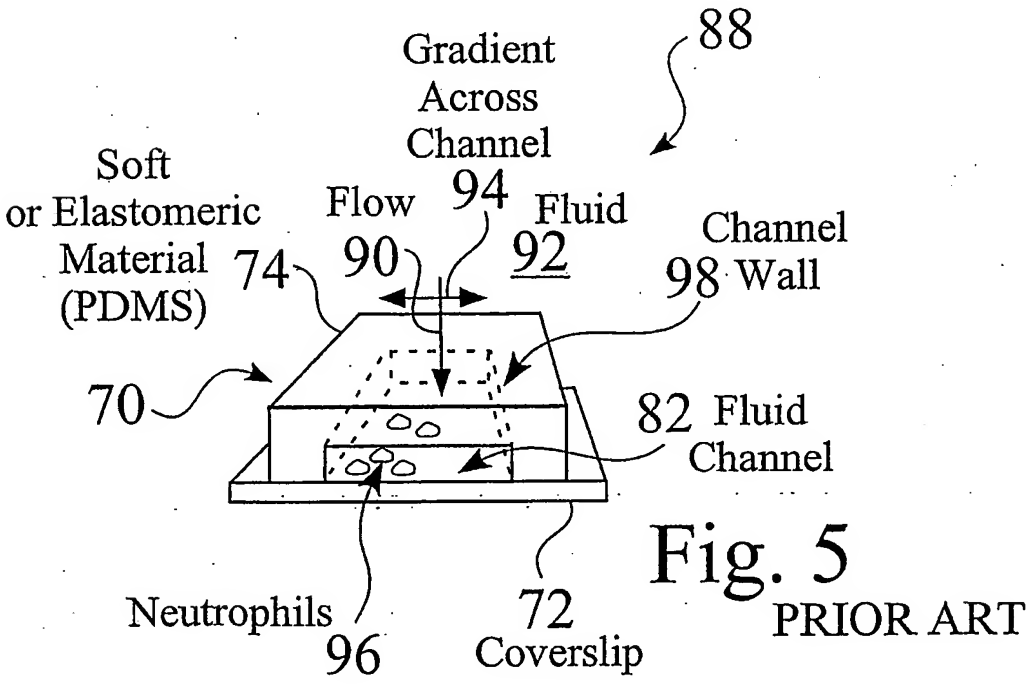
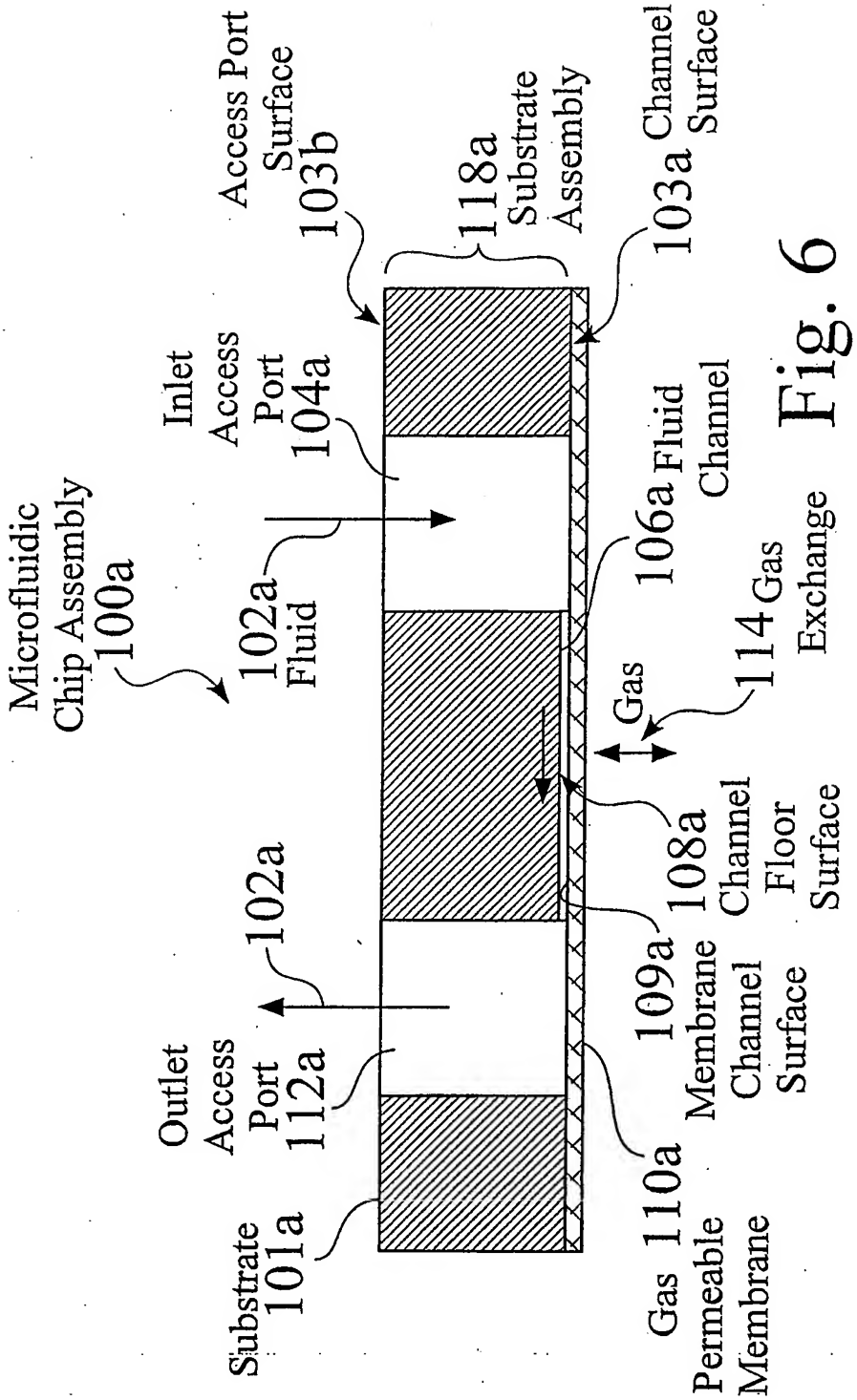


Fig. 5
PRIOR ART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Substrate 101a
Outlet Access Port 112a
102a
Gas Permeable Membrane 110a
Membrane Channel Surface 109a
Channel Floor Surface 108a
Gas Exchange 114
Gas
Fluid Channel 106a
Inlet Access Port 104a
102a Fluid
Access Port Surface 103b
Substrate Assembly 118a
Channel Surface 103a

Fig. 6

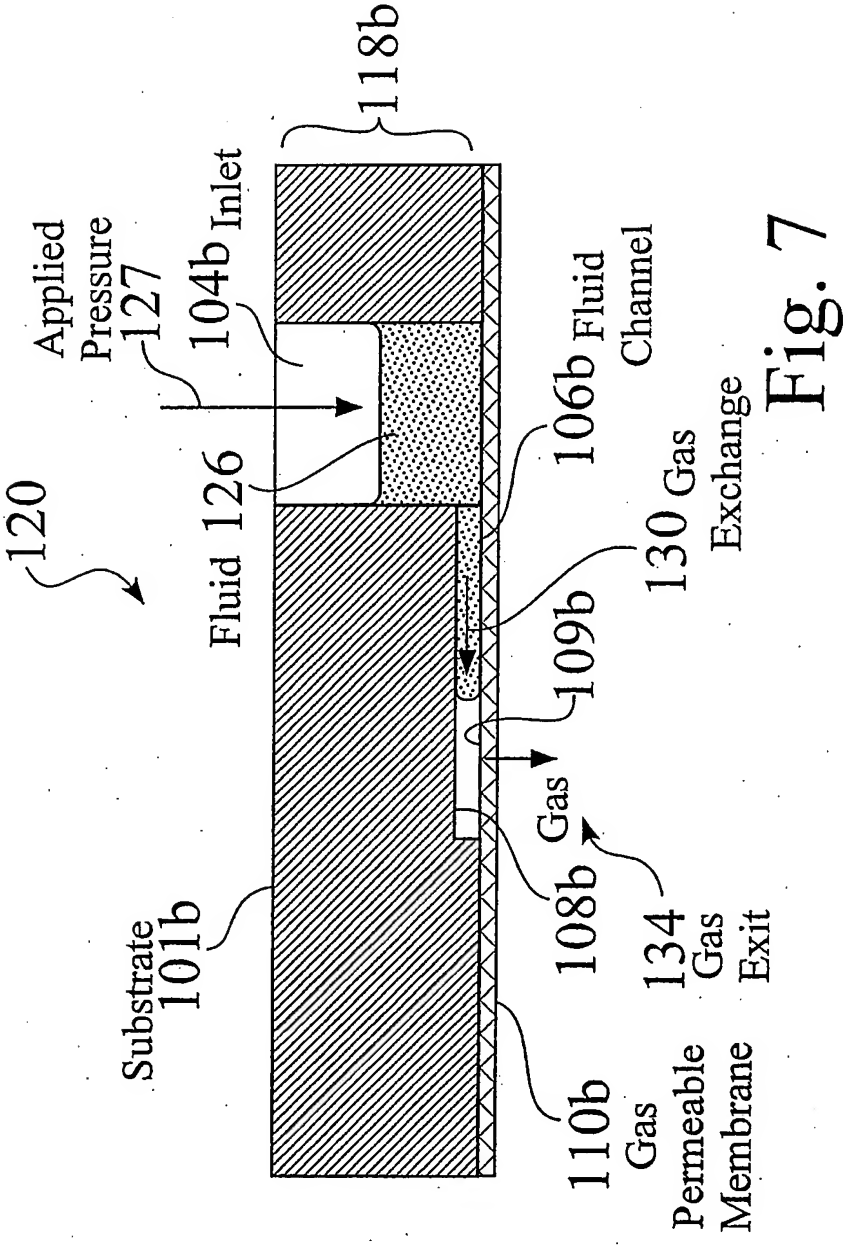
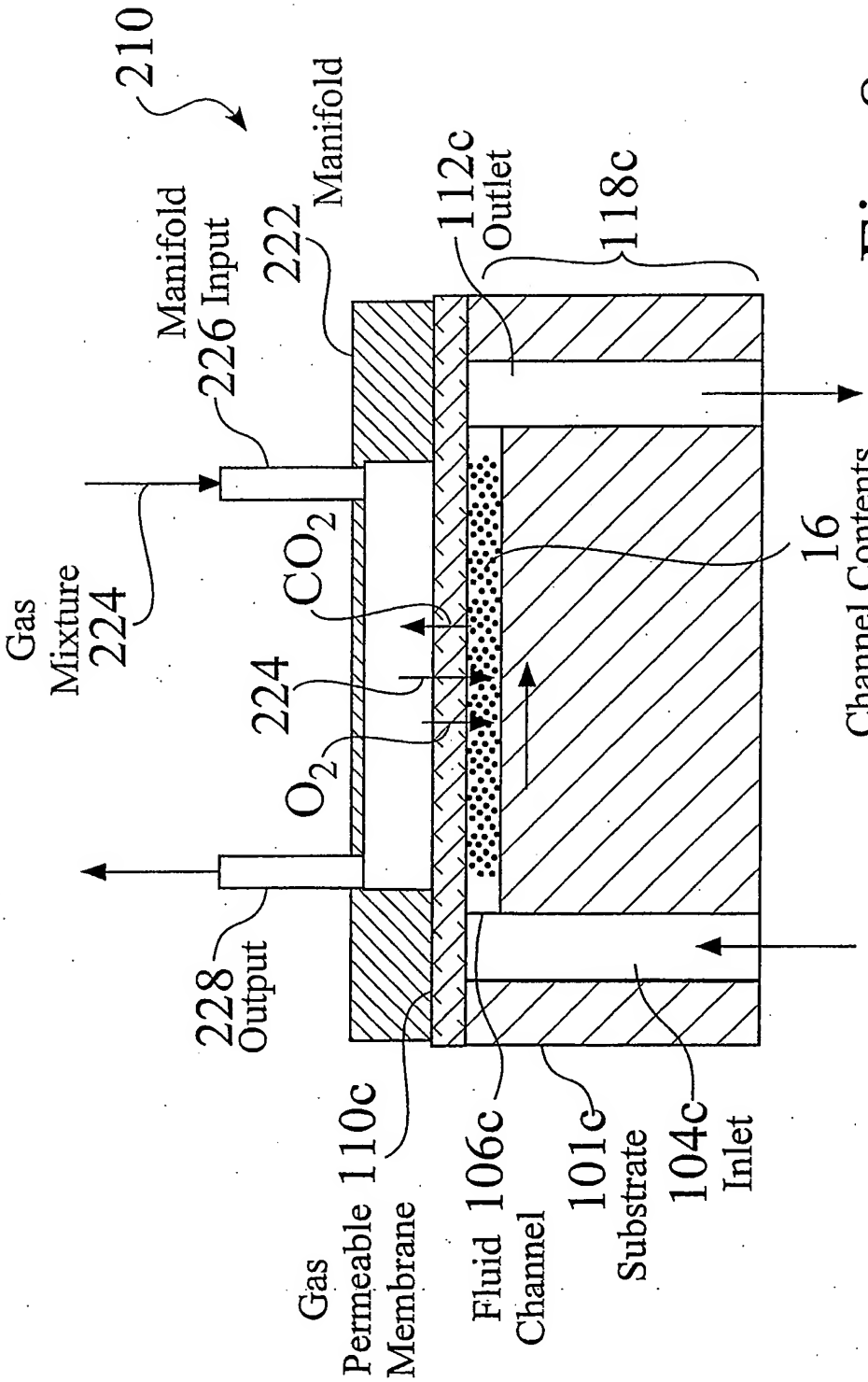

118b
Applied Pressure 127
104b Inlet
Fluid 126
120
106b Fluid Channel
130 Gas Exchange
109b
Gas
108b
134 Gas Exit
Substrate 101b
110b Gas Permeable Membrane

Fig. 7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226 Input
112c
Outlet
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CO_2
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O_2
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Substrate
101c
Inlet
104c

Fig. 8

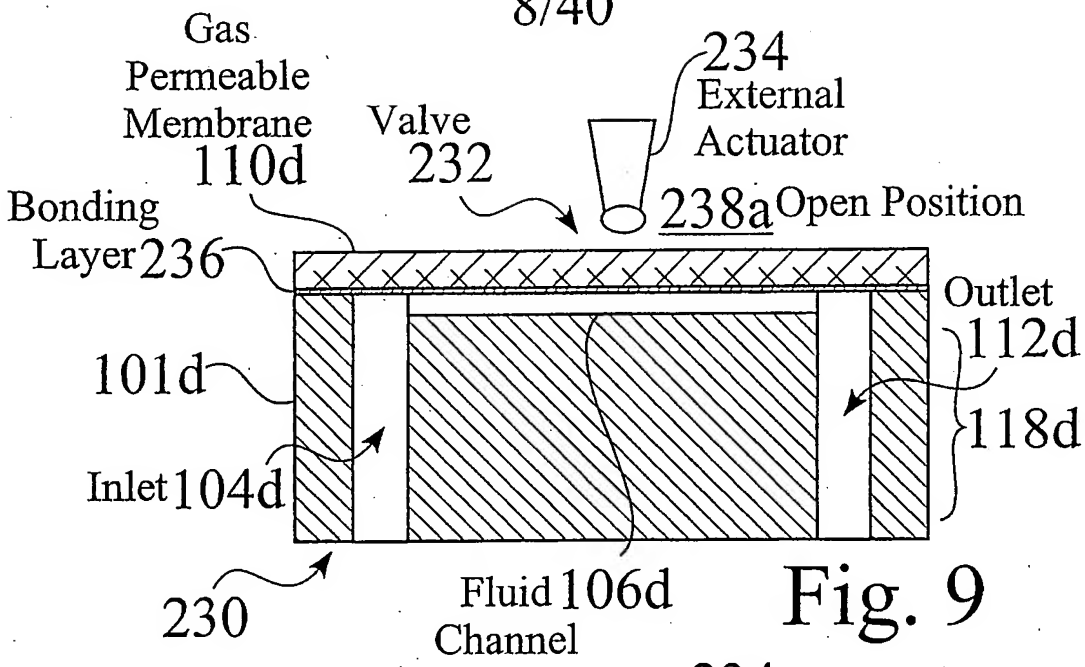


Fig. 9

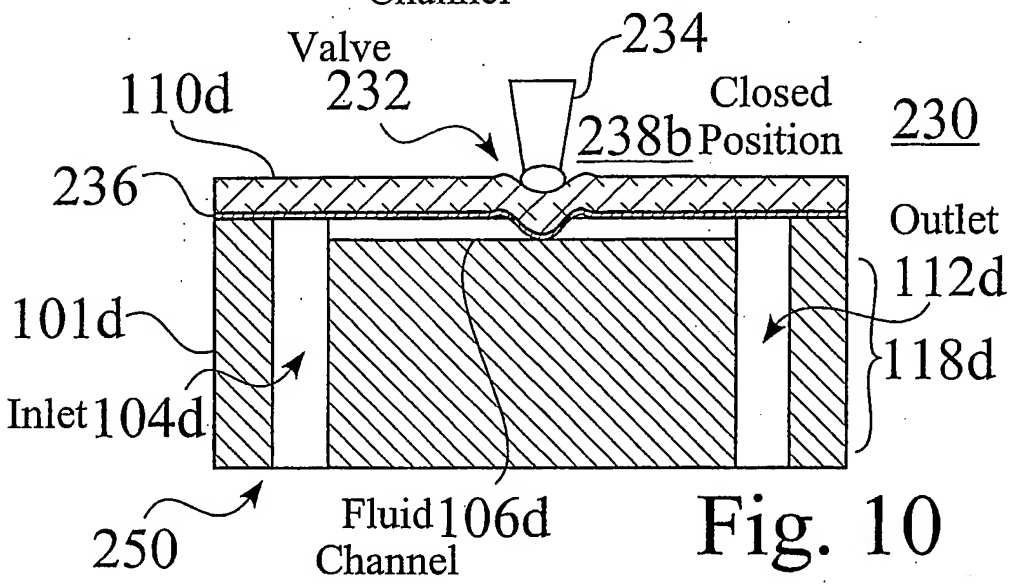


Fig. 10

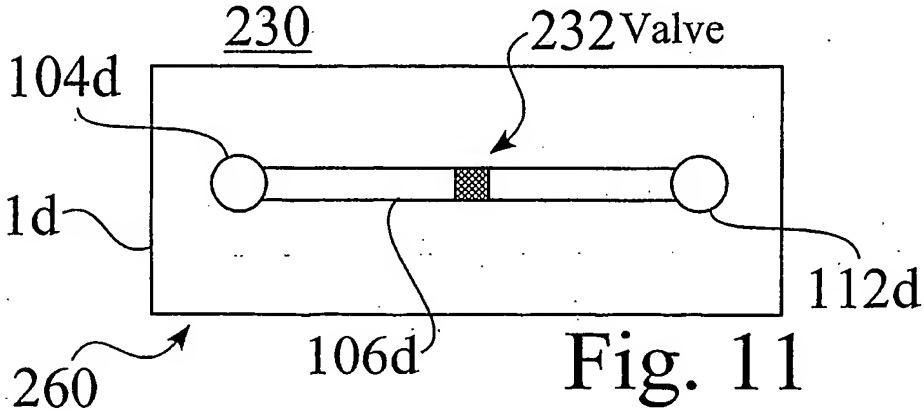


Fig. 11

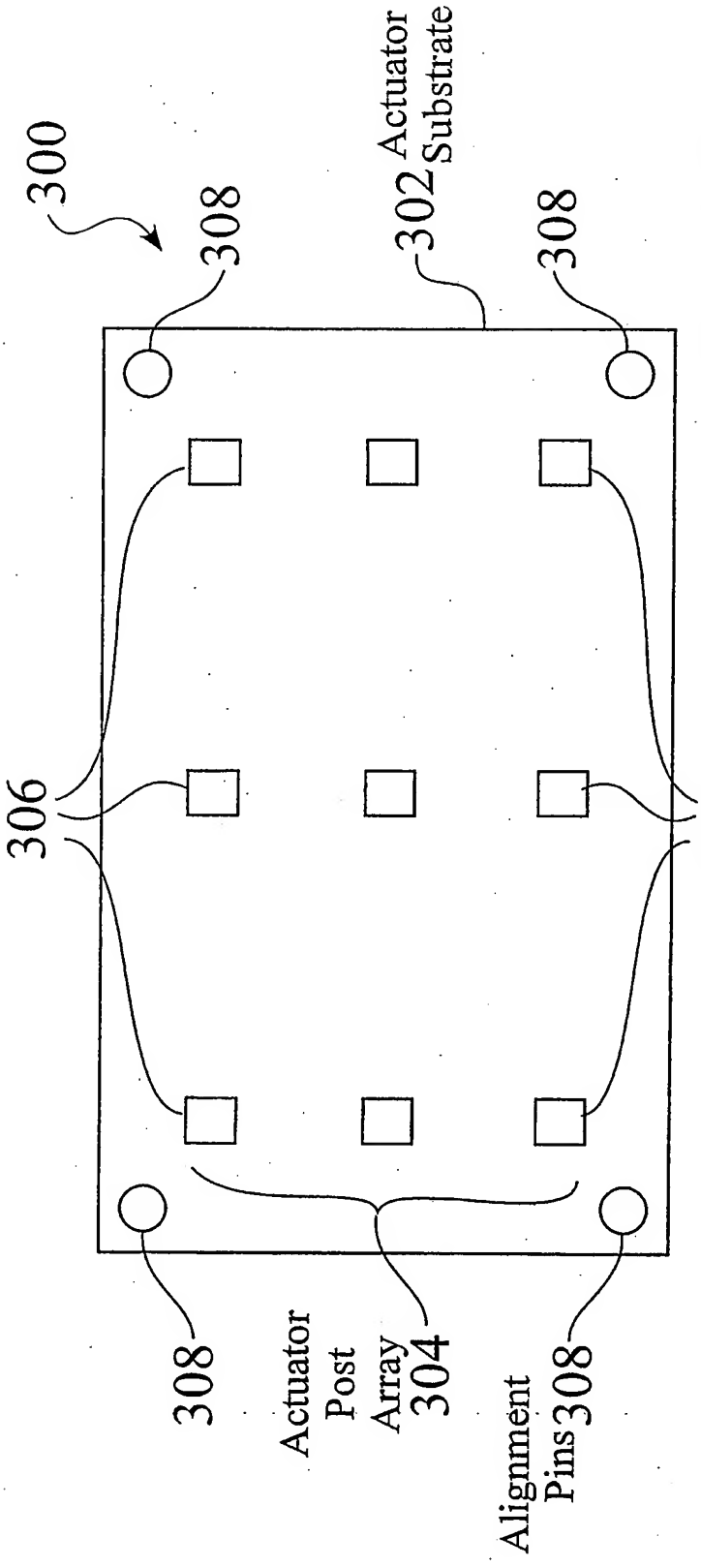
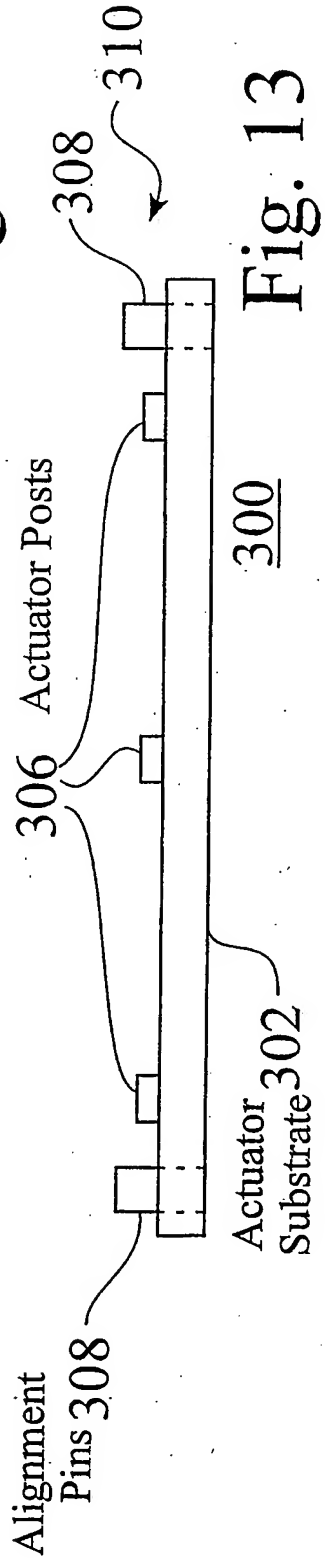

300
308
302
Actuator Substrate
308
306
306
308
Actuator Post Array 304
Alignment Pins 308

Fig. 12


310
308
Actuator Posts
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300
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Actuator Substrate 302
Alignment Pins 308

Fig. 13

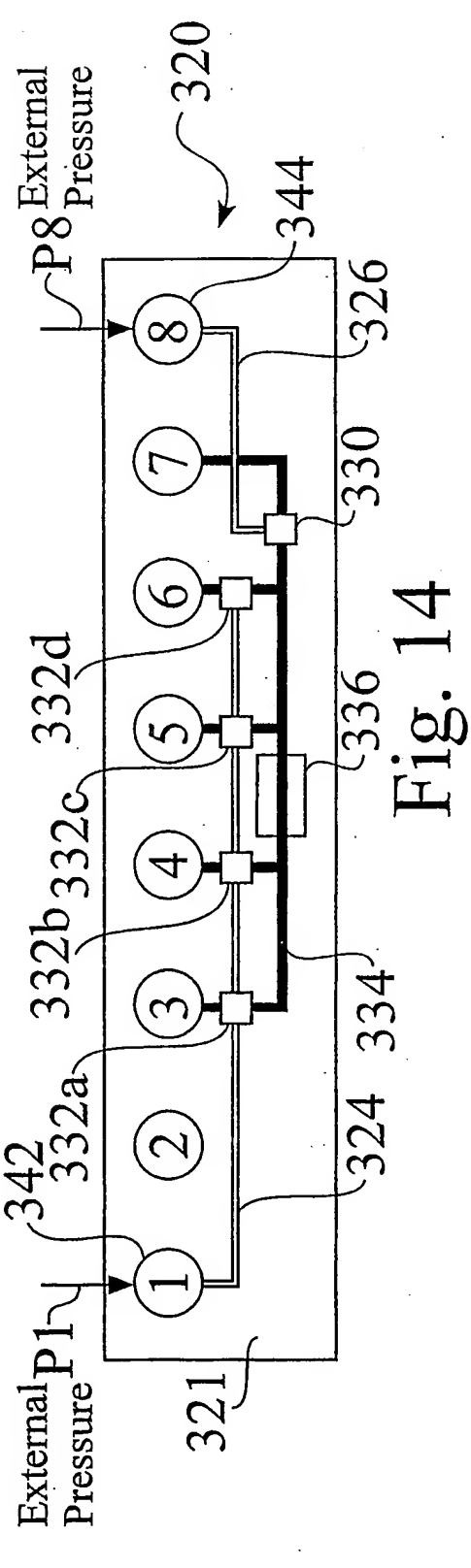


Fig. 14

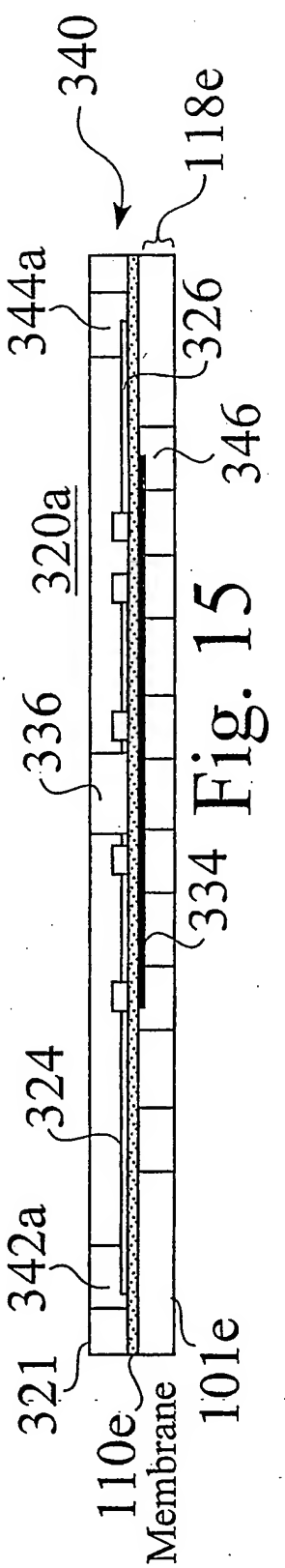


Fig. 15

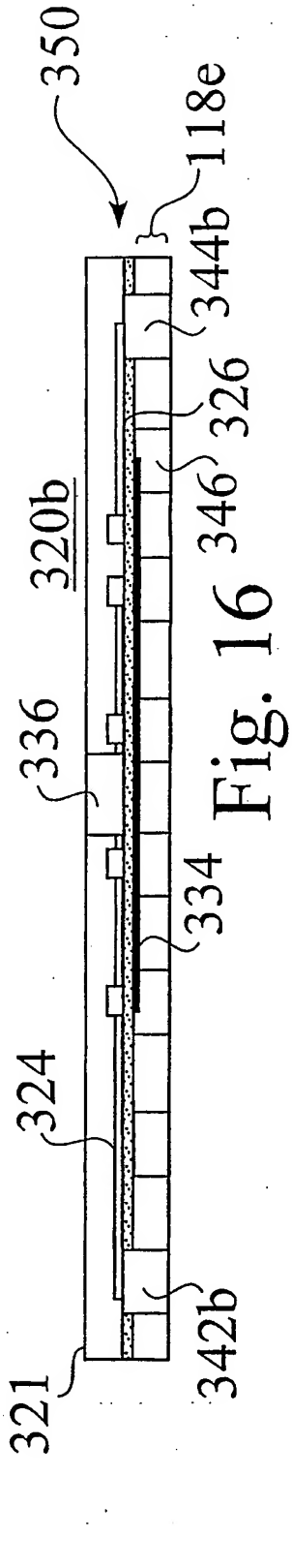


Fig. 16

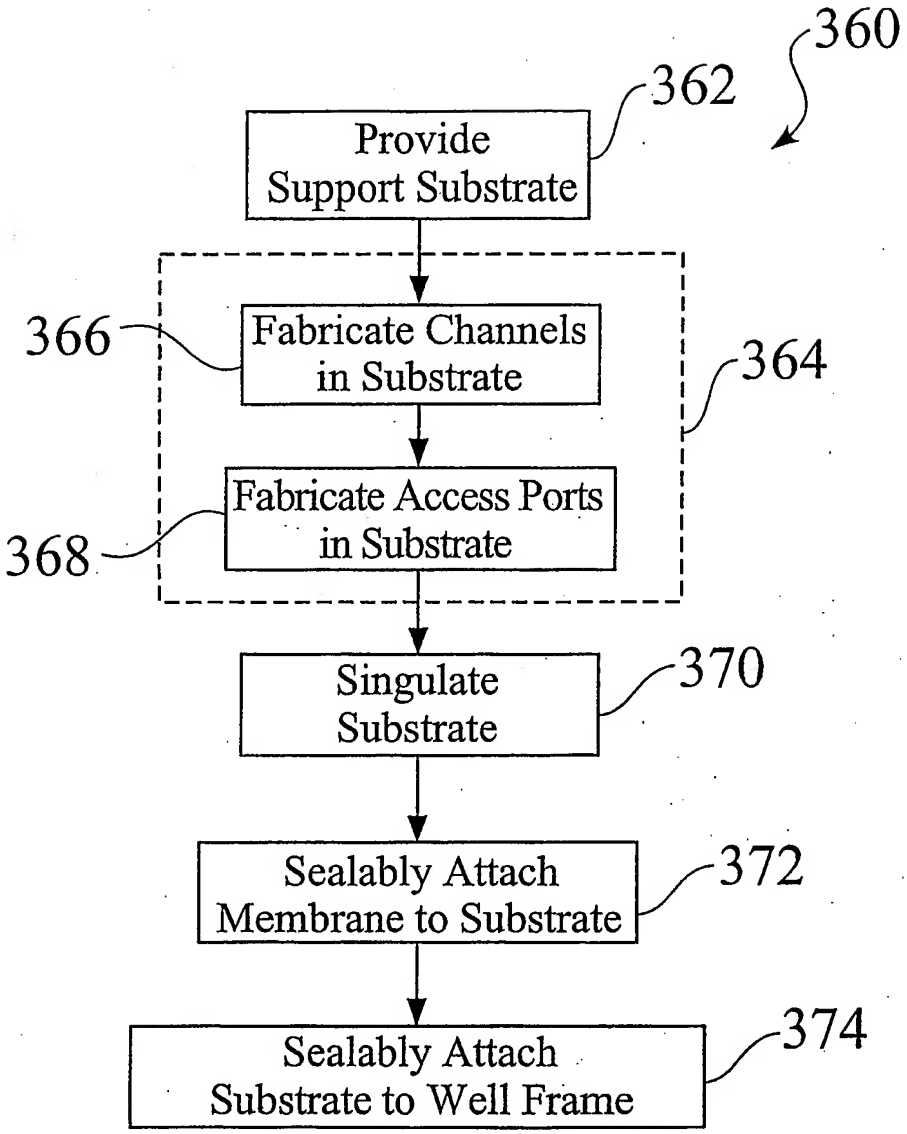

360
362
Provide Support Substrate
366
Fabricate Channels in Substrate
364
Fabricate Access Ports in Substrate
368
Singulate Substrate
370
Sealably Attach Membrane to Substrate
372
Sealably Attach Substrate to Well Frame
374

Fig. 17

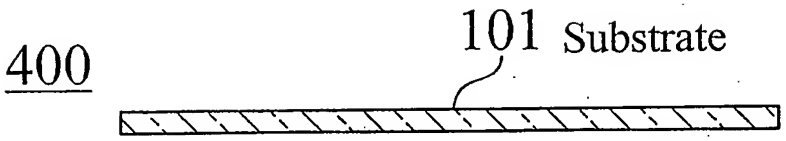


Fig. 18

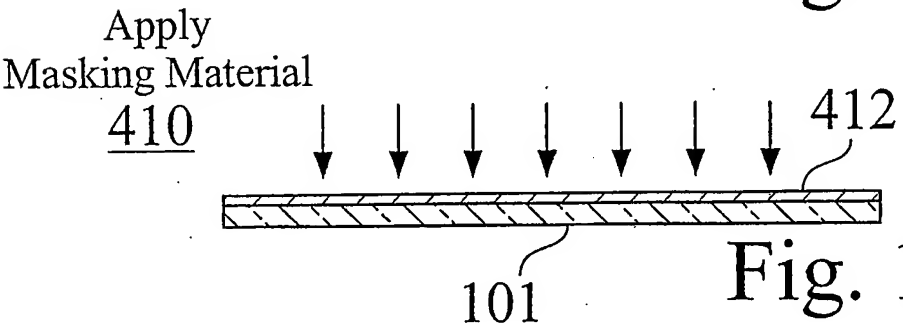


Fig. 19

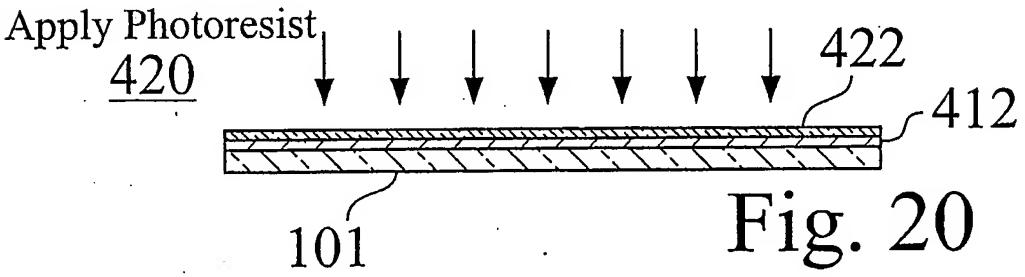


Fig. 20

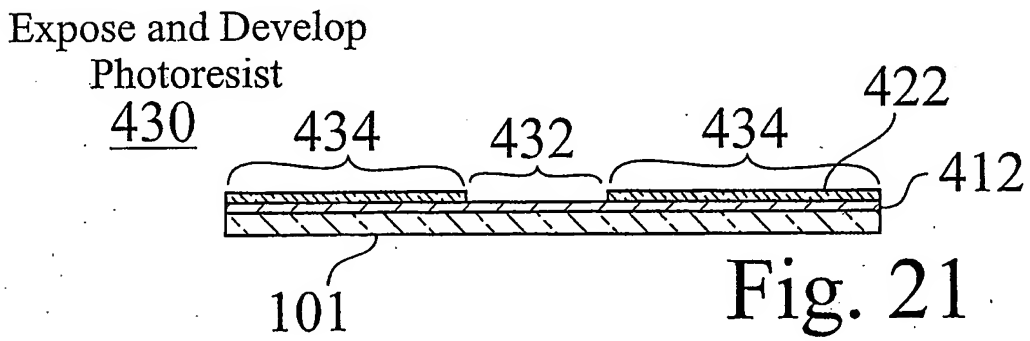


Fig. 21

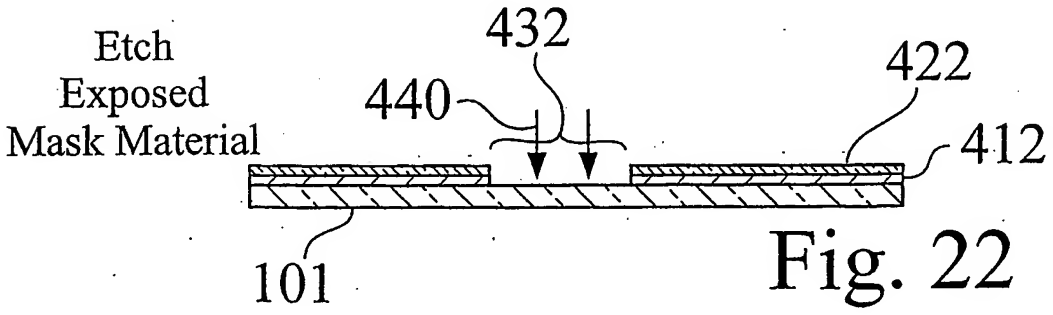


Fig. 22

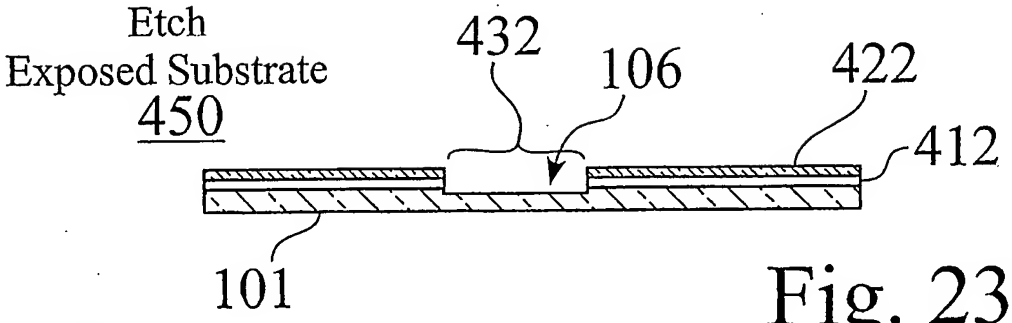


Fig. 23

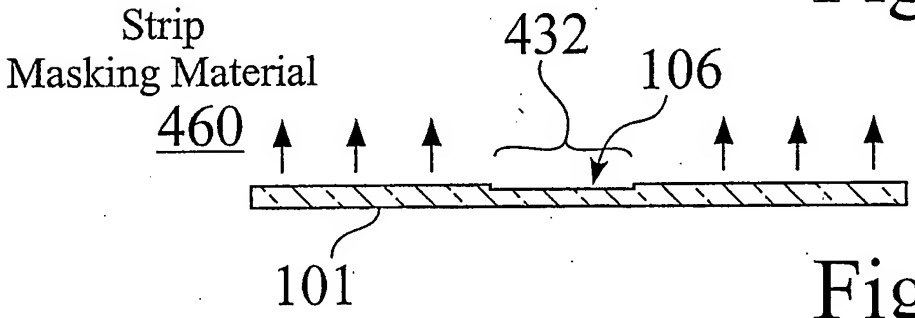


Fig. 24

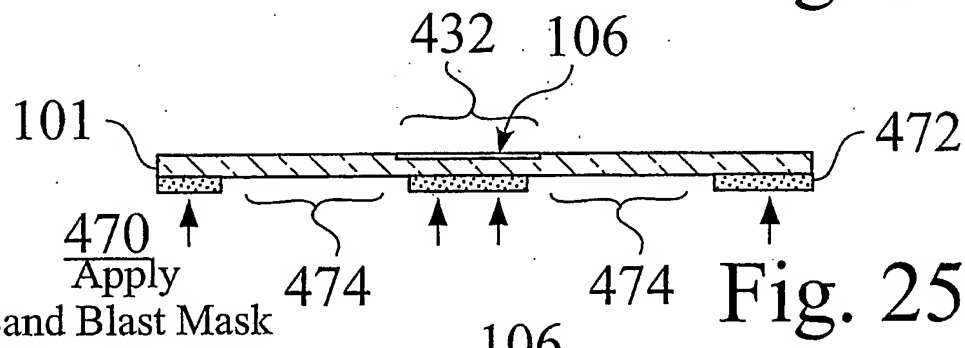


Fig. 25

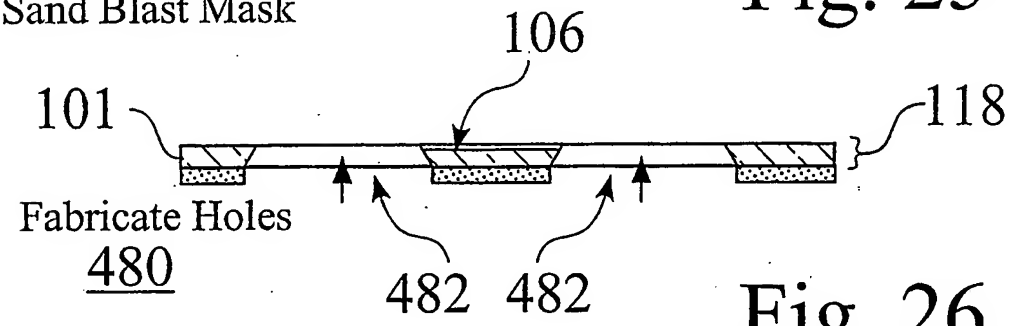


Fig. 26

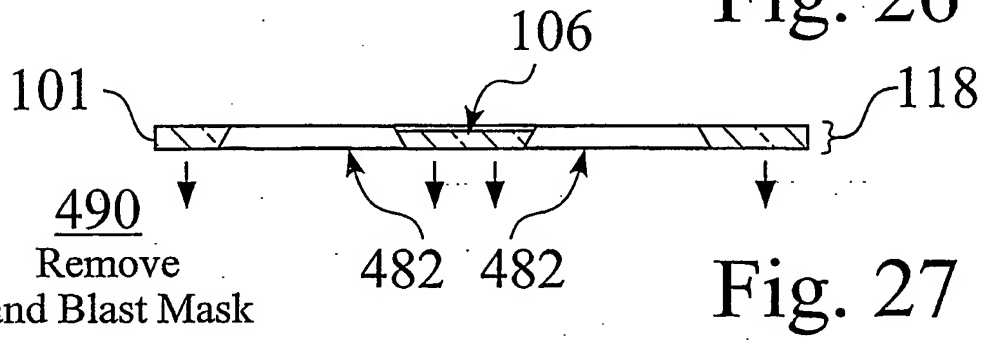


Fig. 27

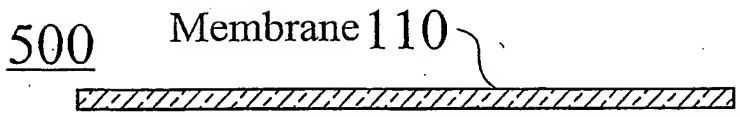


Fig. 28

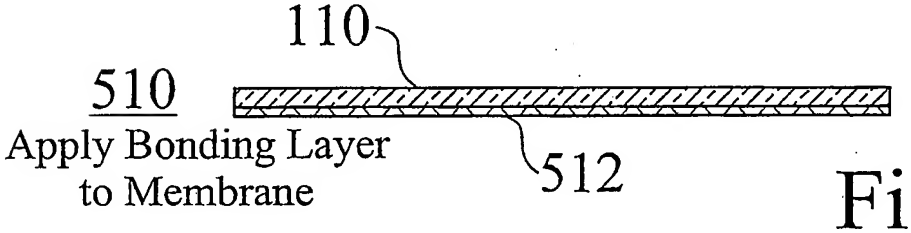


Fig. 29

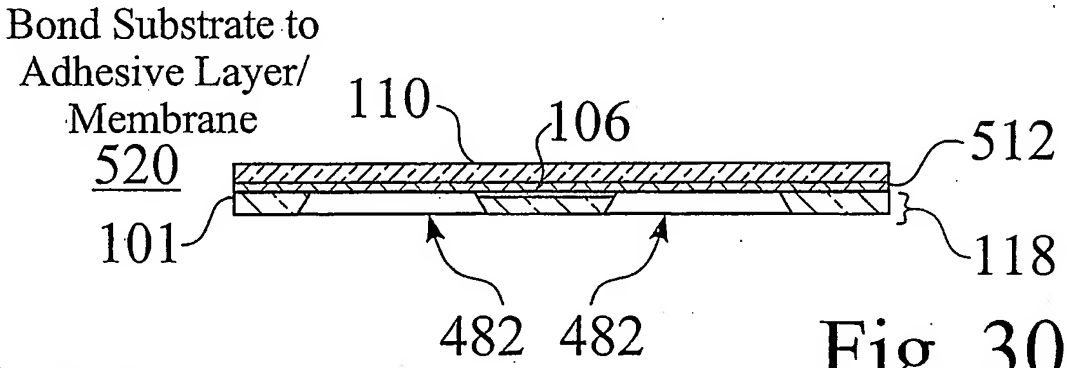


Fig. 30

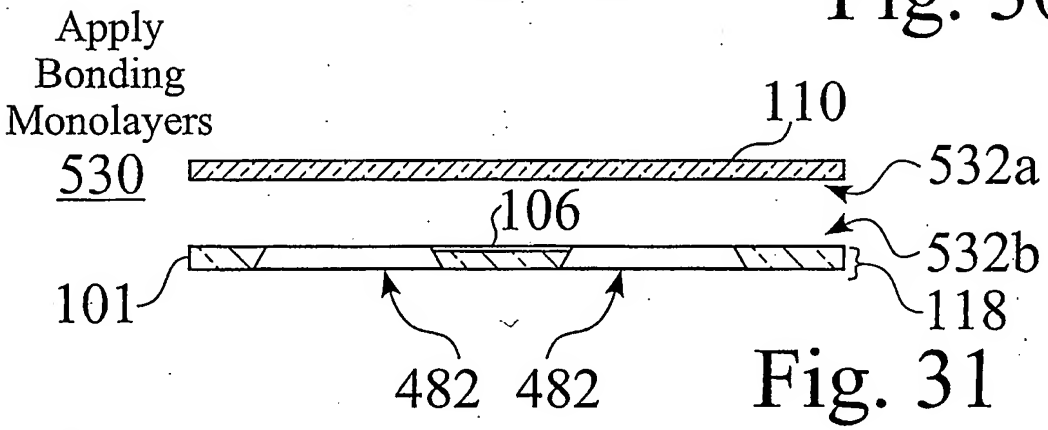


Fig. 31

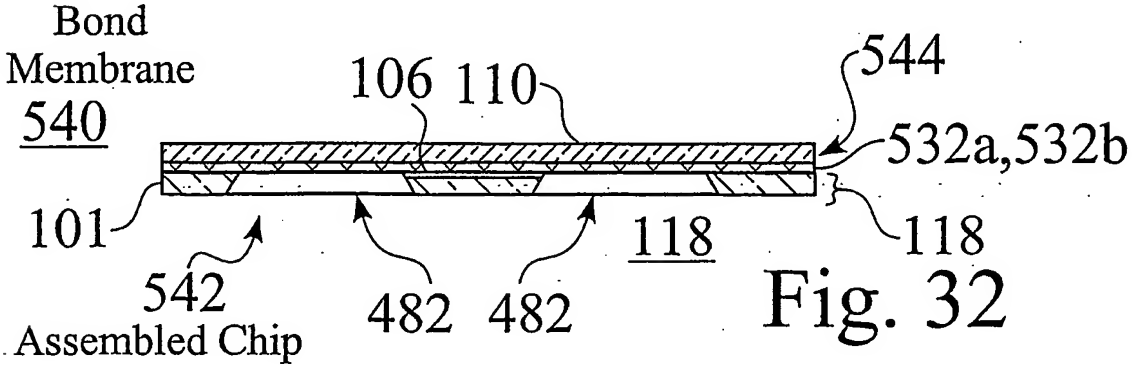


Fig. 32

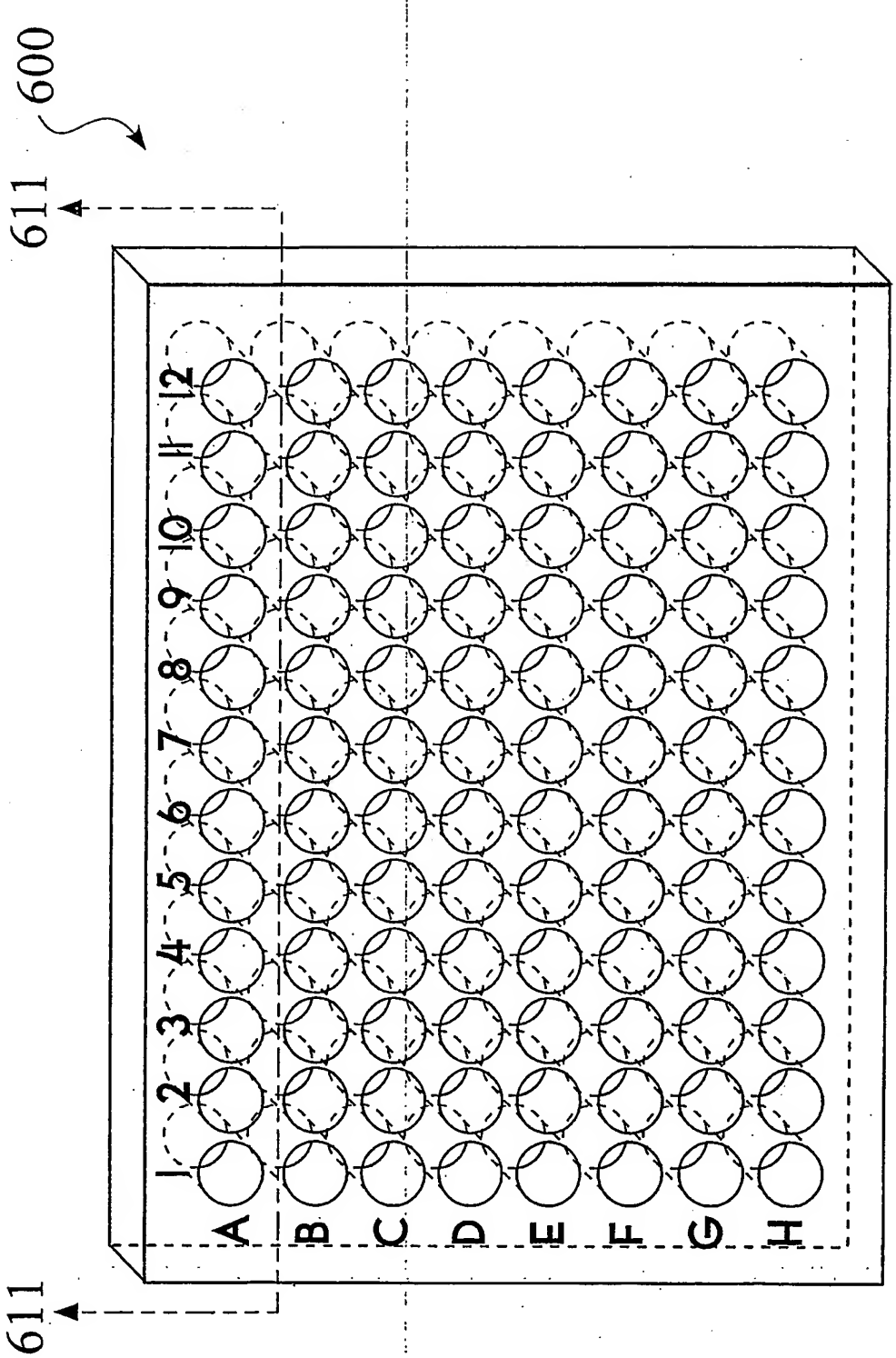


Fig. 33

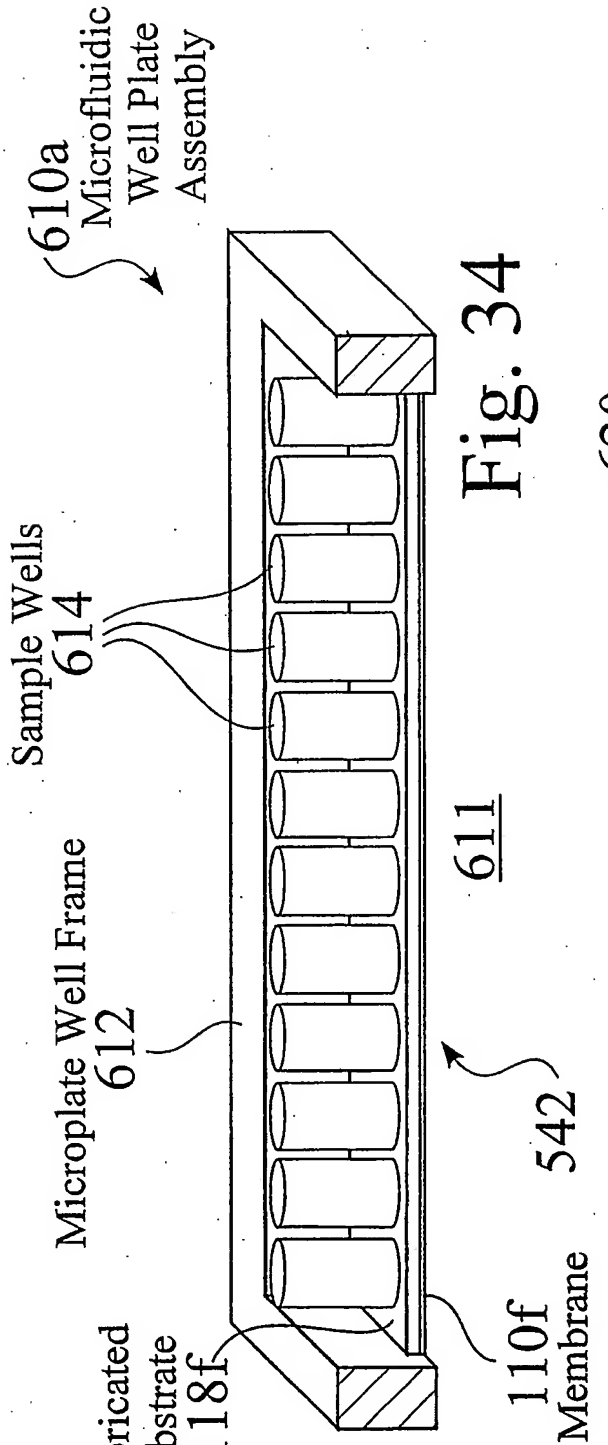

610a
Microfluidic
Well Plate
Assembly
Sample Wells
614
Microplate Well Frame
612
Fabricated
Substrate
118f
611
542
110f
Membrane

Fig. 34

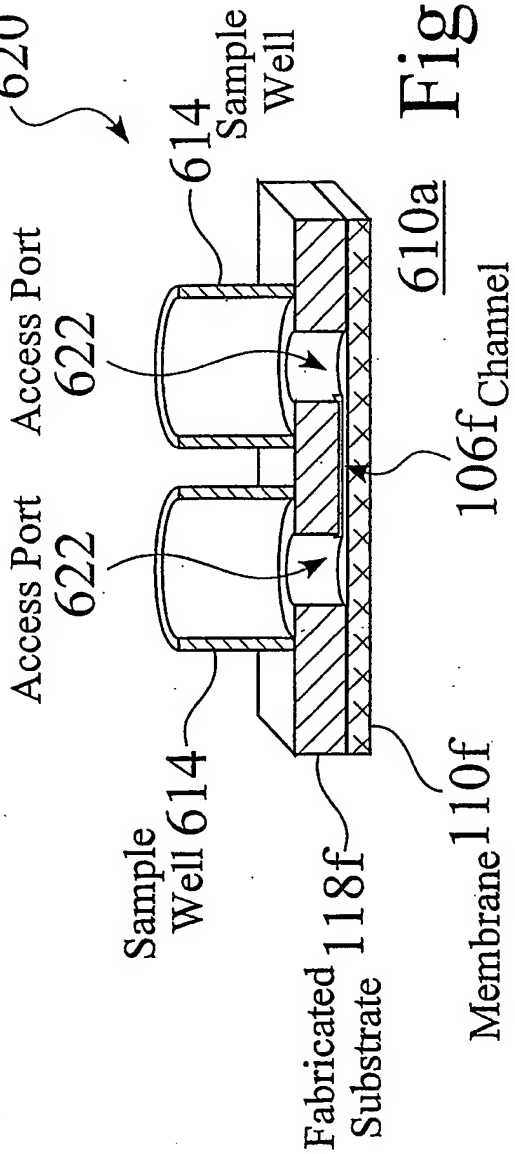

620
614
Sample
Well
Access Port
622
Access Port
622
610a
106f Channel
Sample
Well 614
Fabricated
Substrate
118f
Membrane 110f

Fig. 35

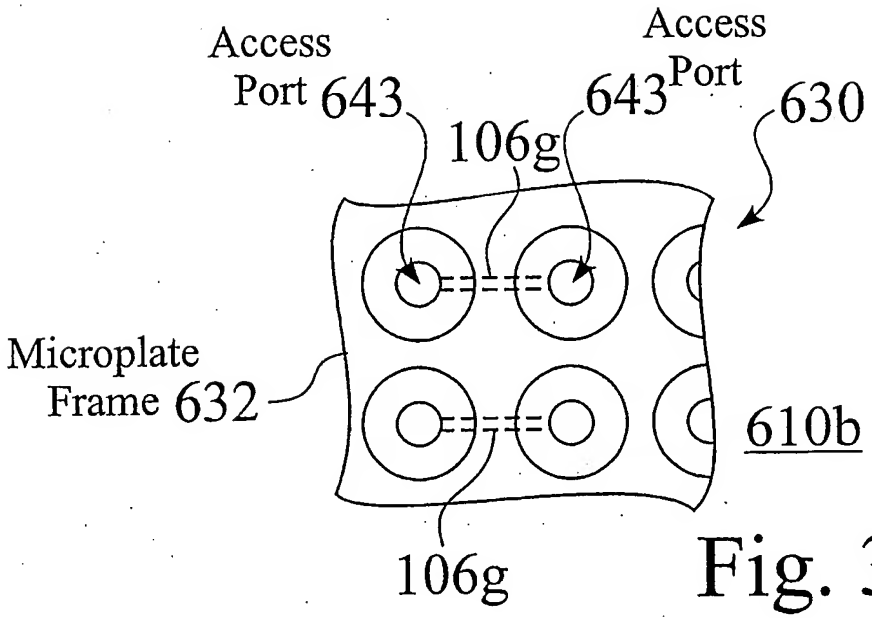

Access
Port 643
643 Access
Port
630
106g
Microplate
Frame 632
610b
106g

Fig. 36

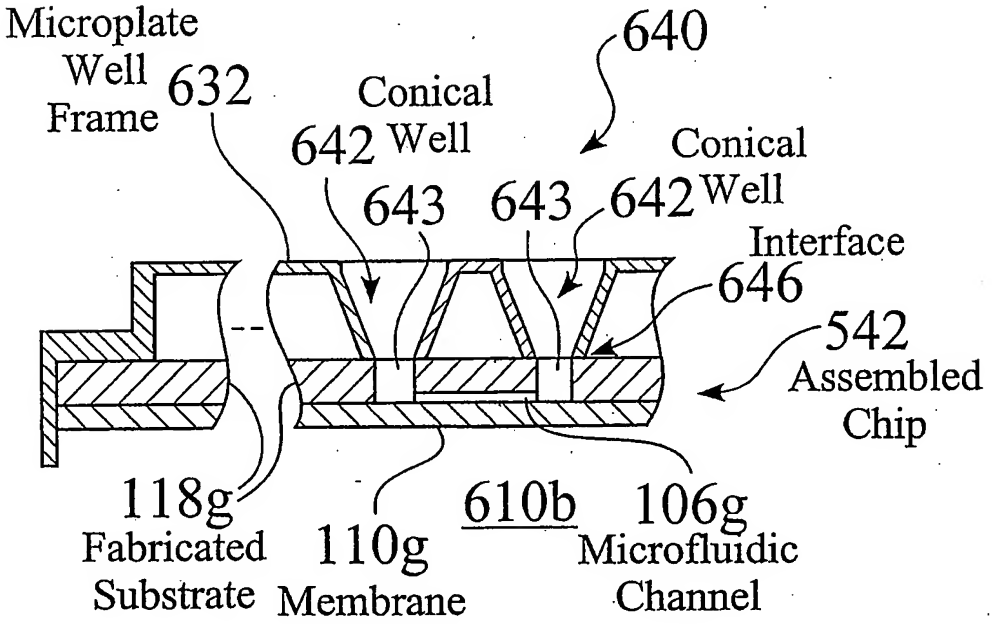

Microplate
Well
Frame 632
640
Conical
642 Well
643
643
642 Conical
Well
Interface
646
542
Assembled
Chip
118g
Fabricated
Substrate
110g
Membrane
610b
106g
Microfluidic
Channel

Fig. 37

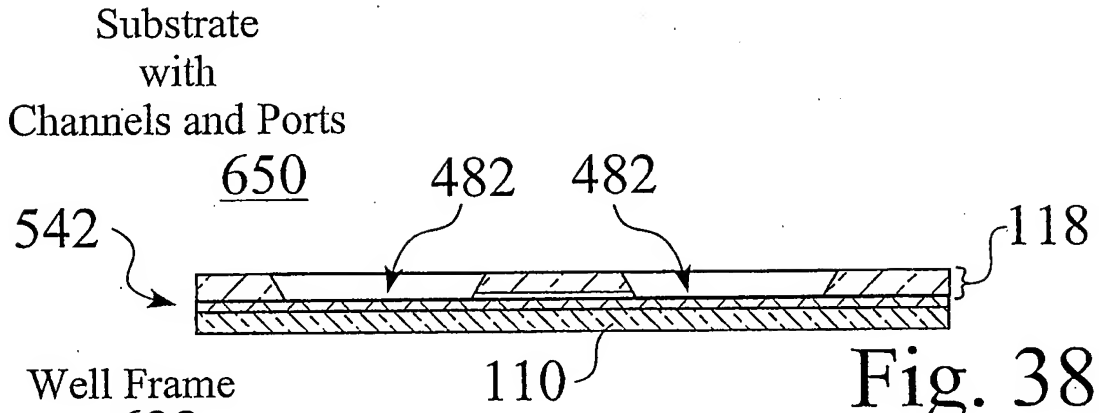


Fig. 38

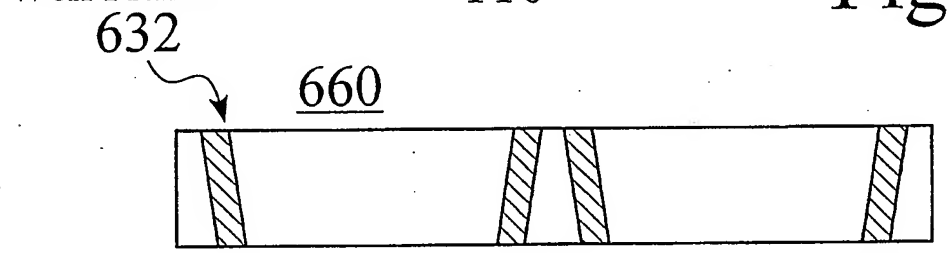


Fig. 39

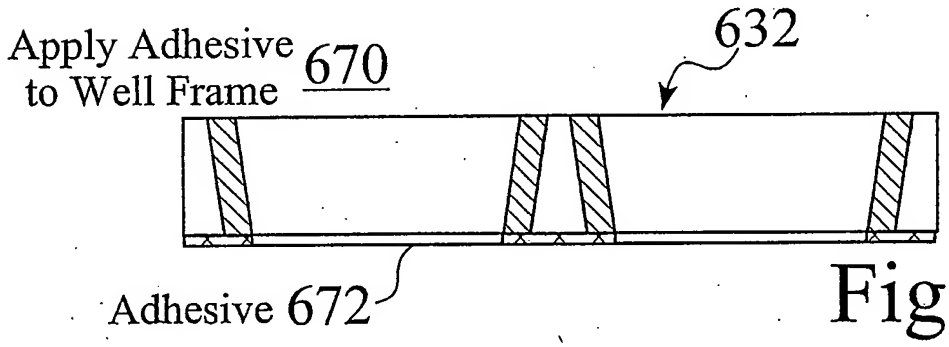


Fig. 40

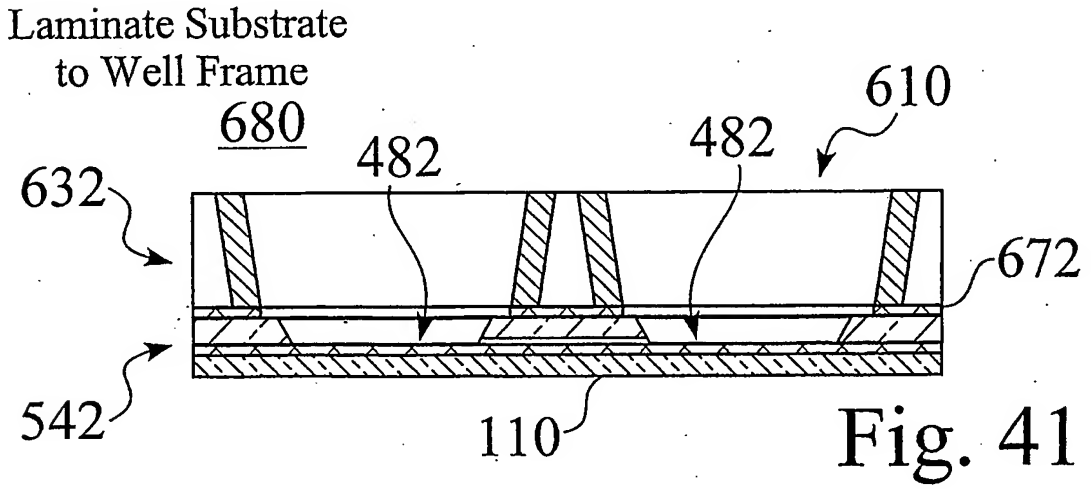


Fig. 41

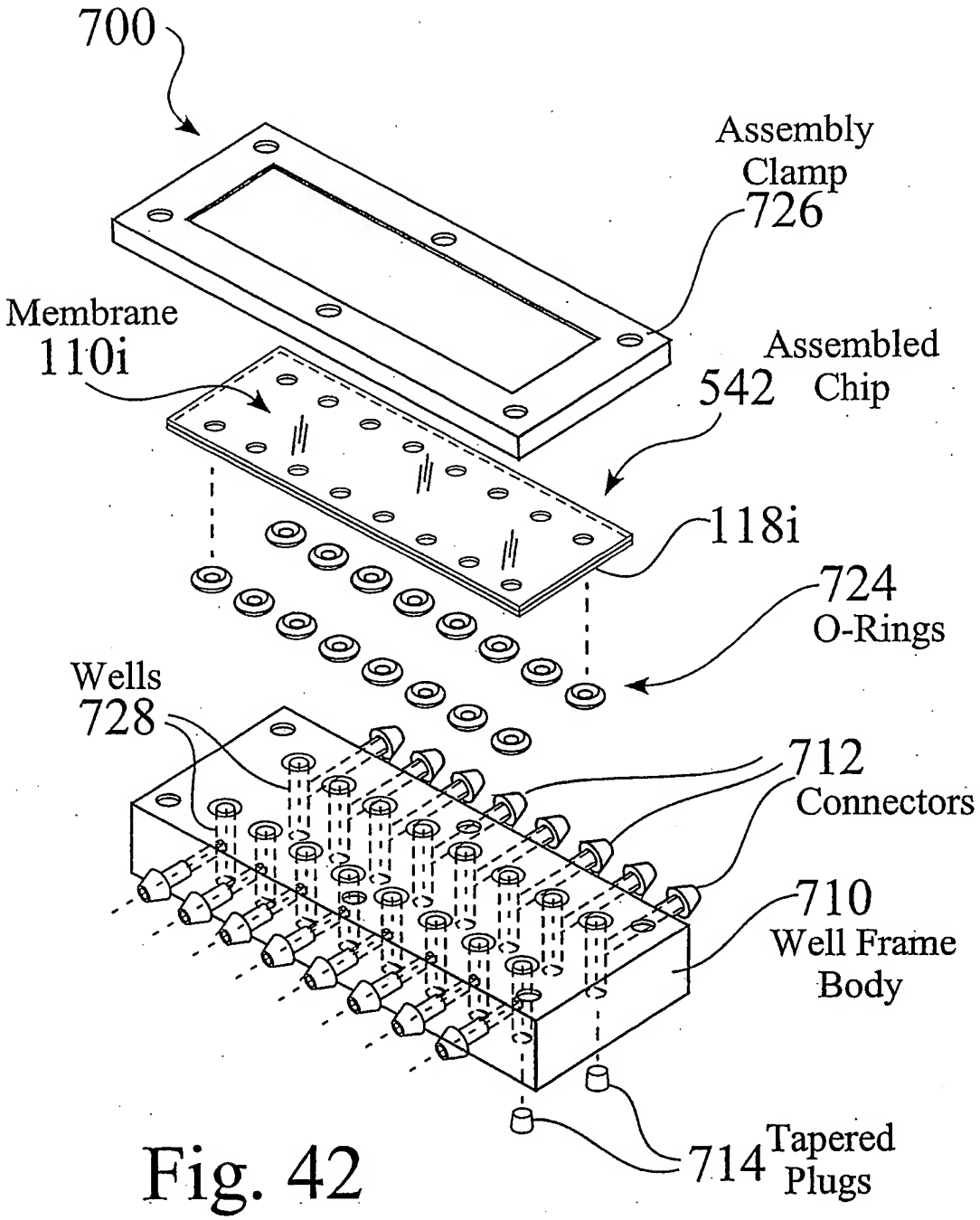
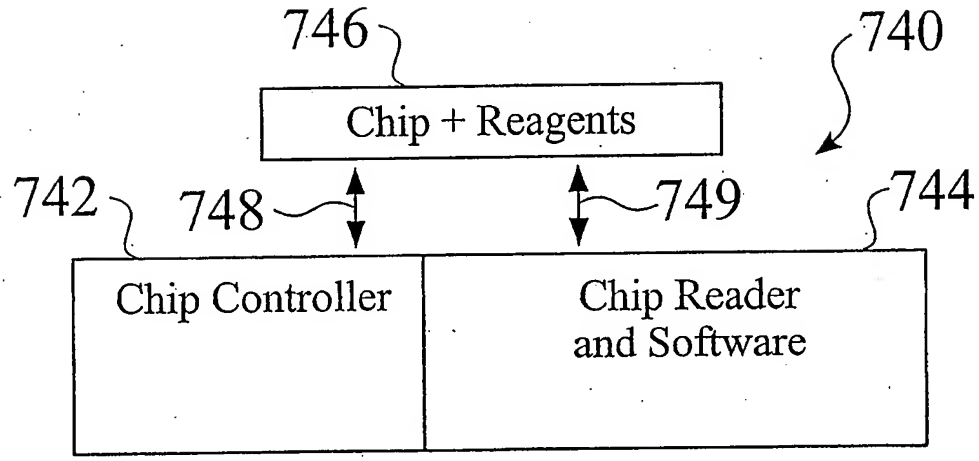

700
Assembly Clamp 726
Membrane 110i
Assembled Chip 542
118i
724 O-Rings
Wells 728
712 Connectors
710 Well Frame Body
714 Tapered Plugs

Fig. 42



System Architecture

Fig. 43

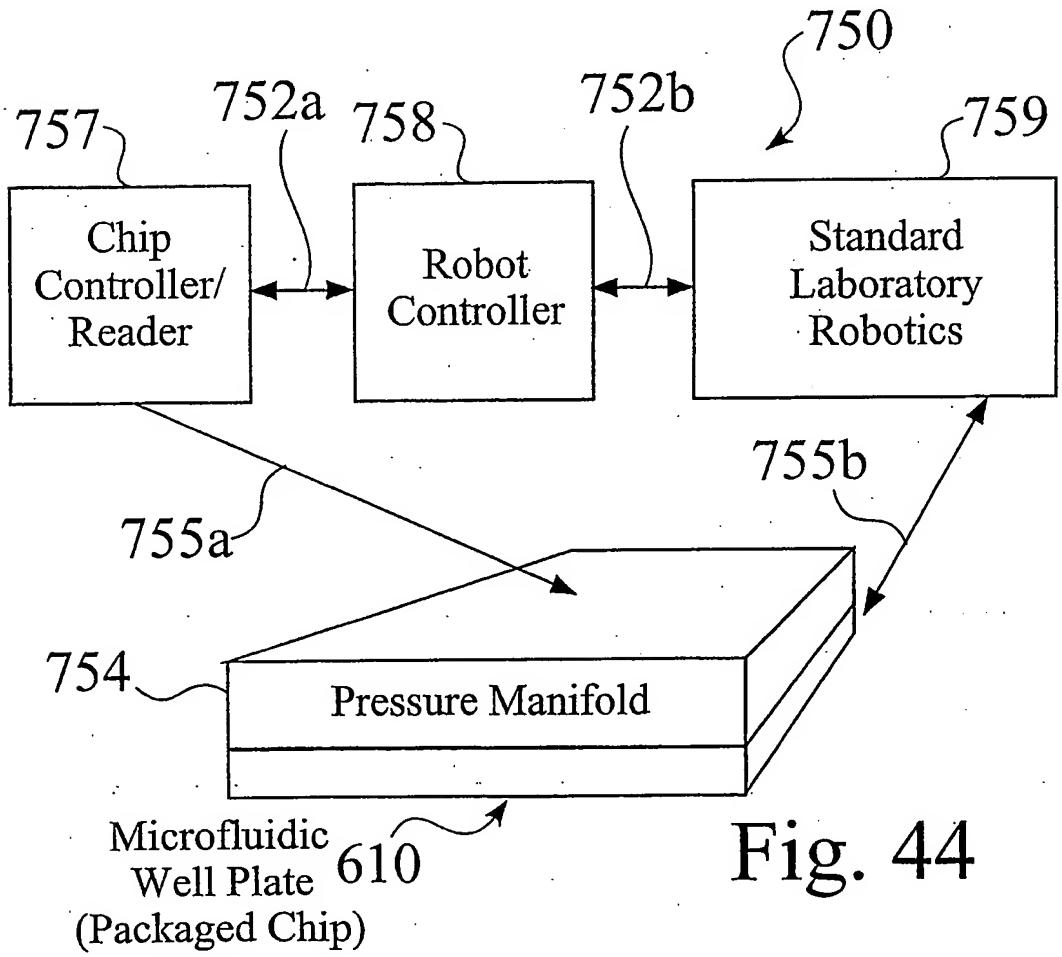


Fig. 44

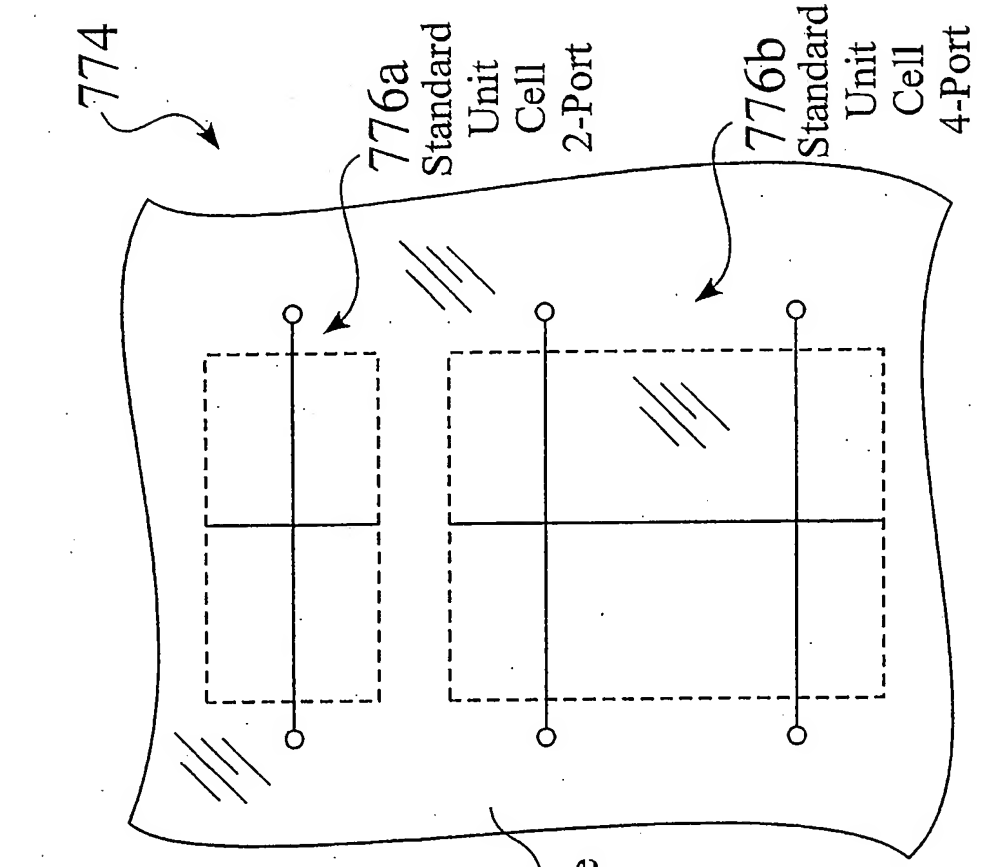

774
776a Standard Unit Cell 2-Port
776b Standard Unit Cell 4-Port
762 Substrate

Fig. 46

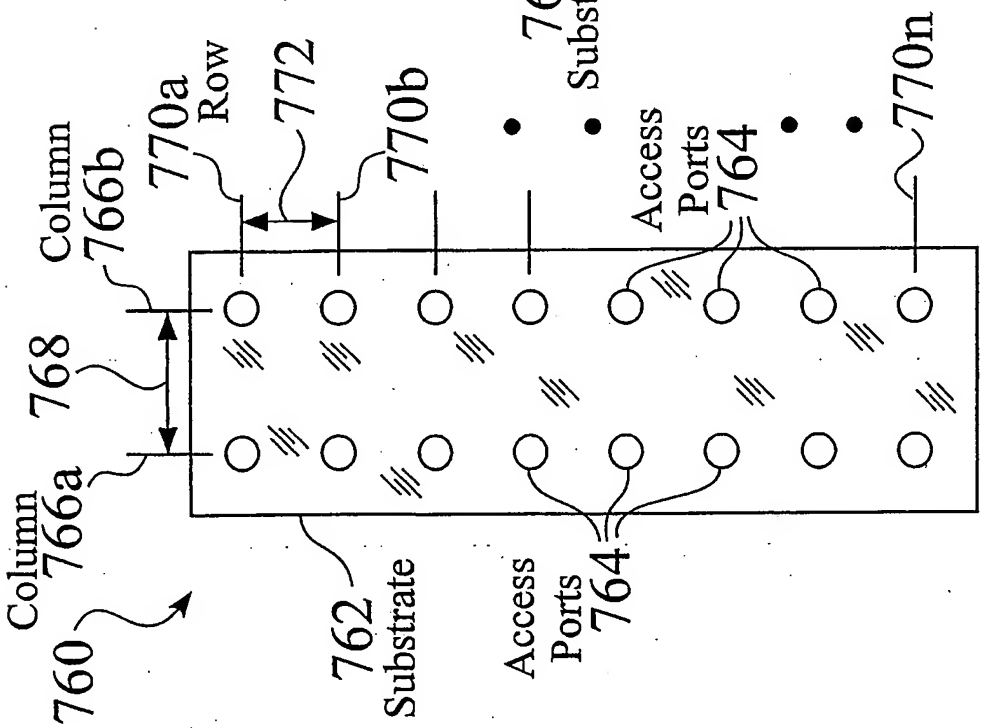

760
Column 766a
768
Column 766b
770a Row
772
770b
770n
Access Ports 764
762 Substrate
Access Ports 764

Fig. 45

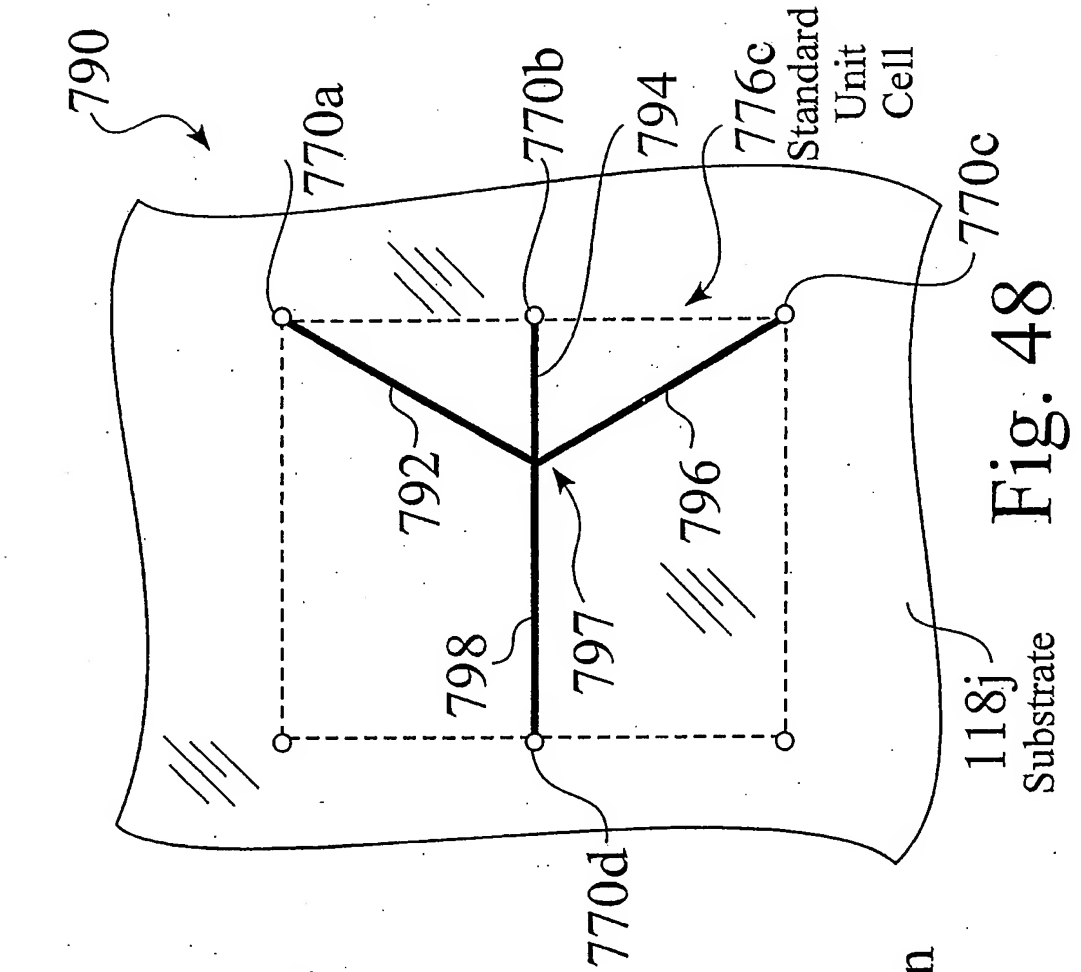

790
770a
770b
794
776c
Standard
Unit
Cell
770c
792
796
798
797
118j
Substrate
770d

Fig. 48

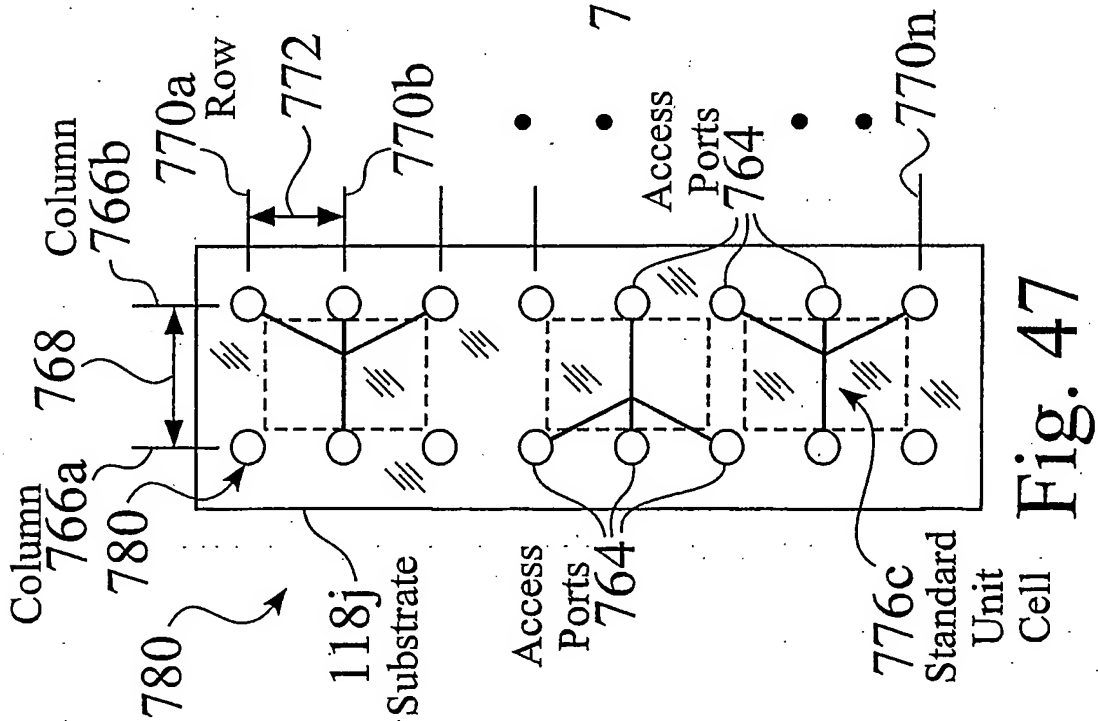

Column
766b
770a
Row
772
770b
Access
Ports
764
770n
768
Column
766a
780
780
118j
Substrate
Access
Ports
764
776c
Standard
Unit
Cell

Fig. 47

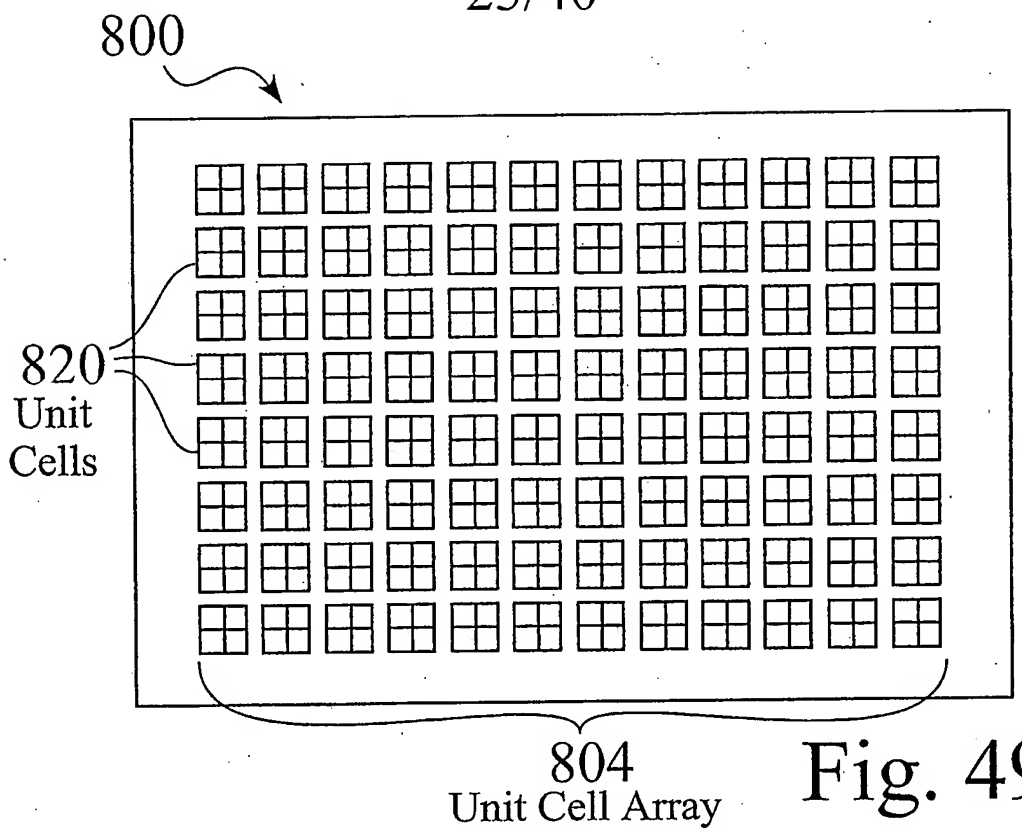

800
820
Unit
Cells
804
Unit Cell Array

Fig. 49

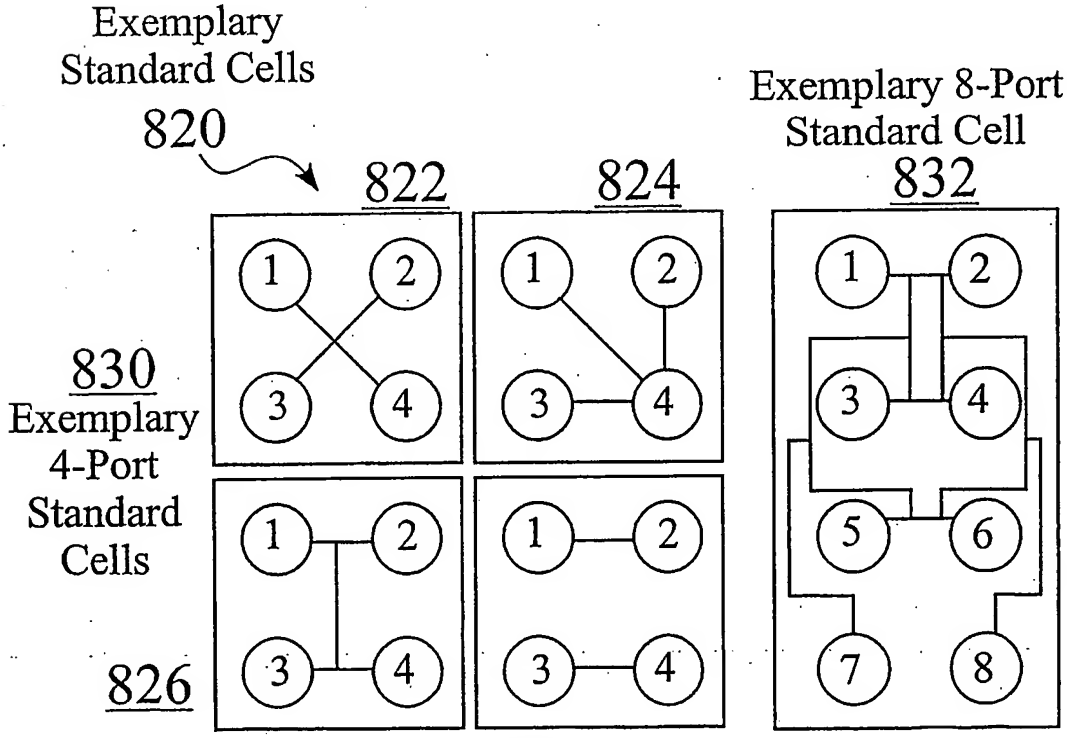

Exemplary
Standard Cells
820
822
824
Exemplary 8-Port
Standard Cell
832
830
Exemplary
4-Port
Standard
Cells
826
828
1
2
3
4
5
6
7
8

Fig. 50

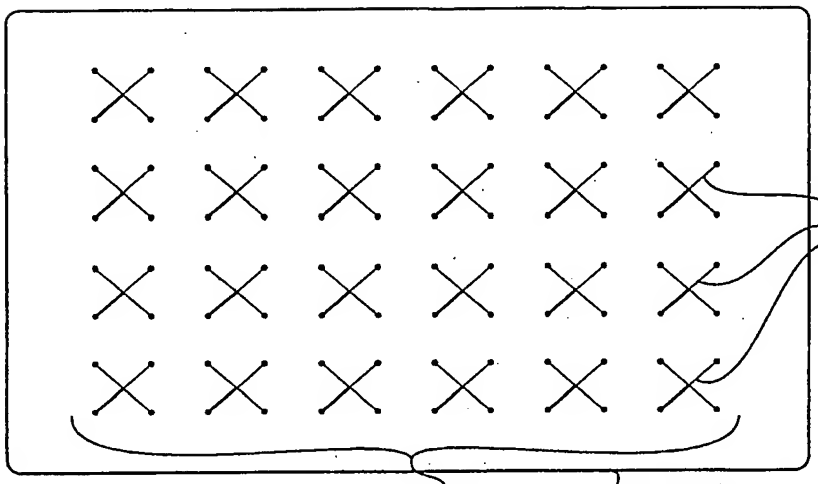


Fig. 51

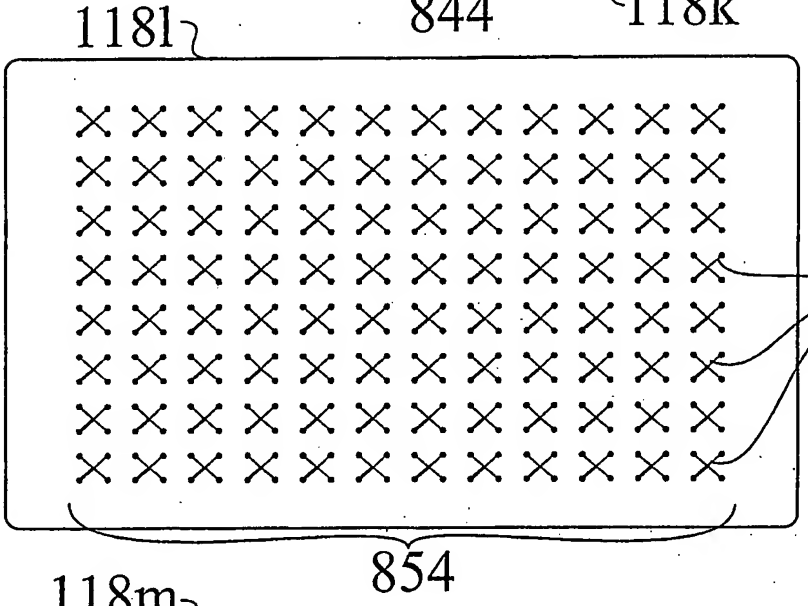


Fig. 52

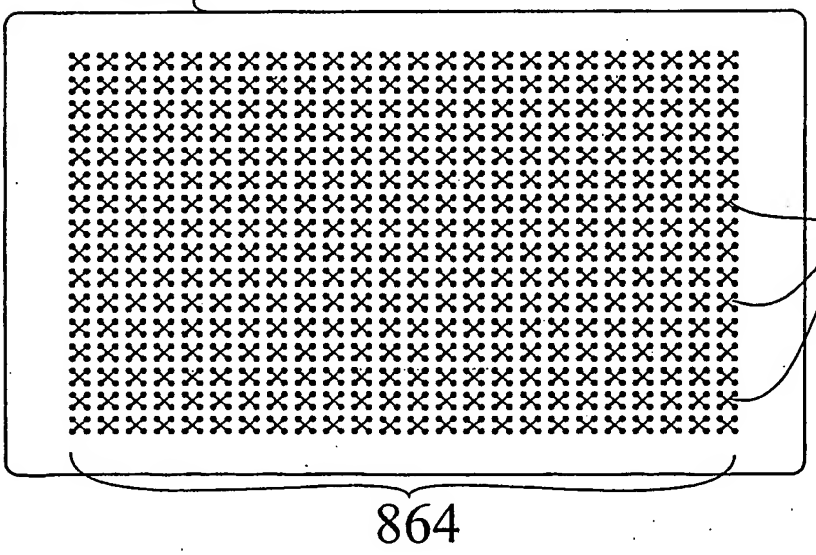


Fig. 53

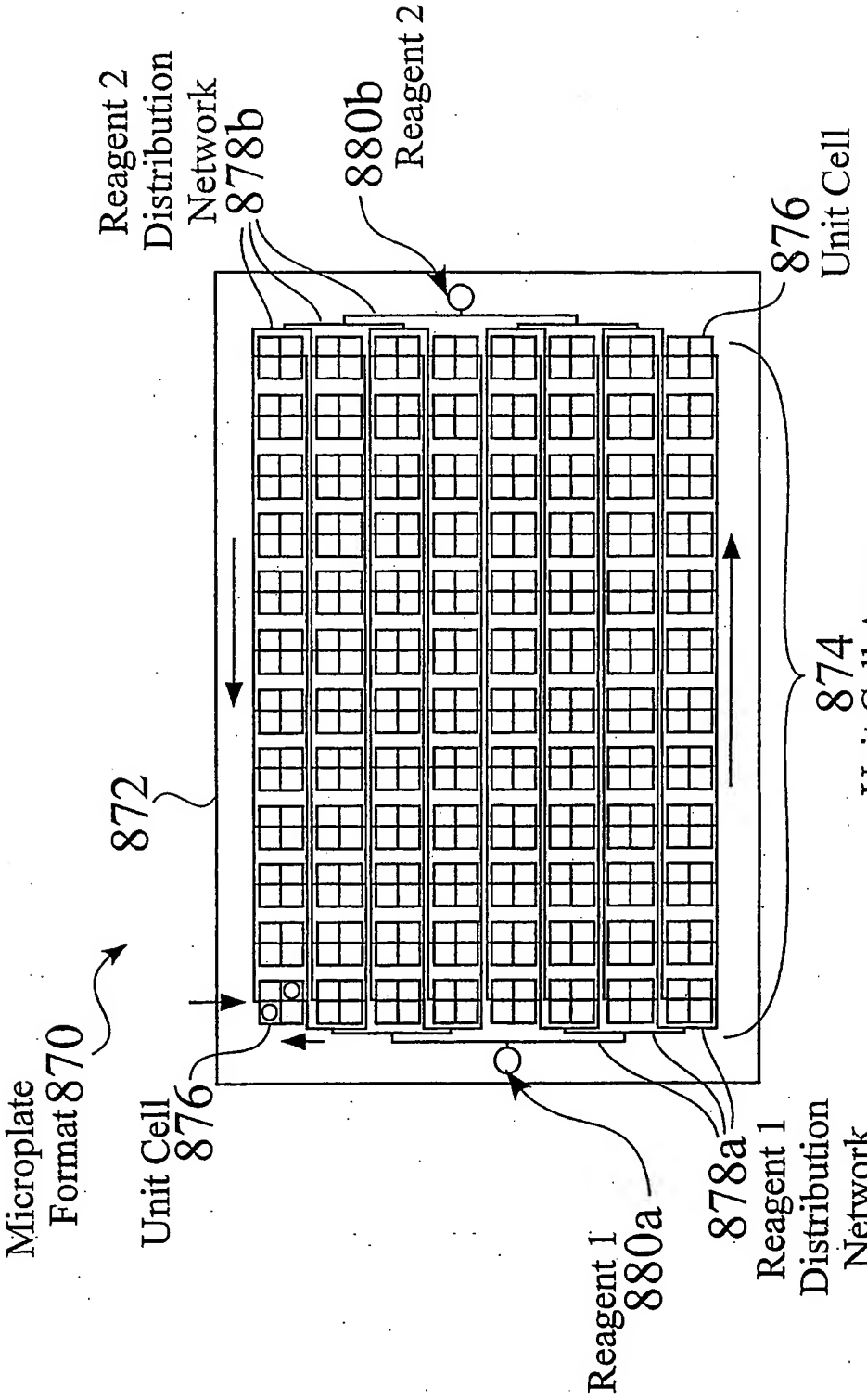

Reagent 2 Distribution Network 878b
880b Reagent 2
876 Unit Cell
872
874 Unit Cell Array
Microplate Format 870
Unit Cell 876
Reagent 1 880a
878a Reagent 1 Distribution Network

Fig. 54

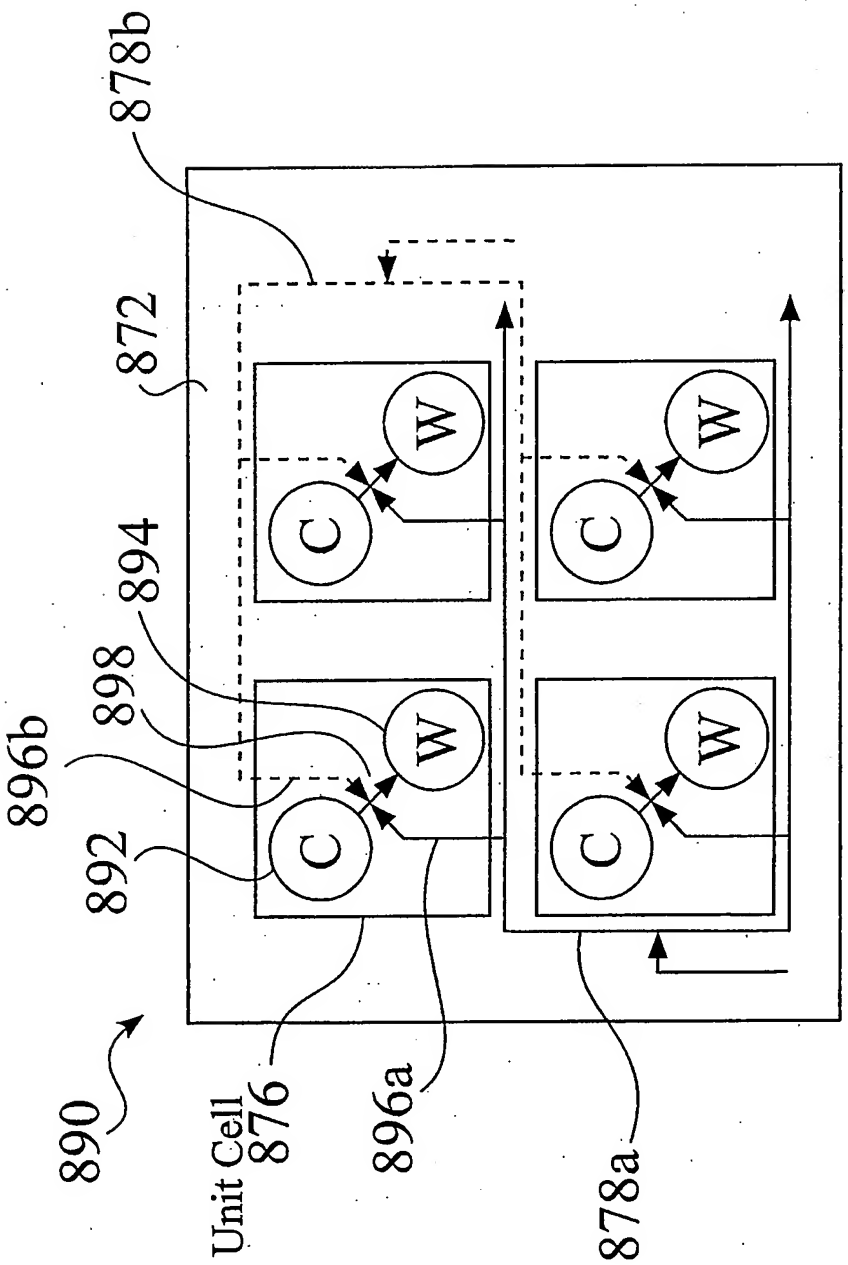


Fig. 55

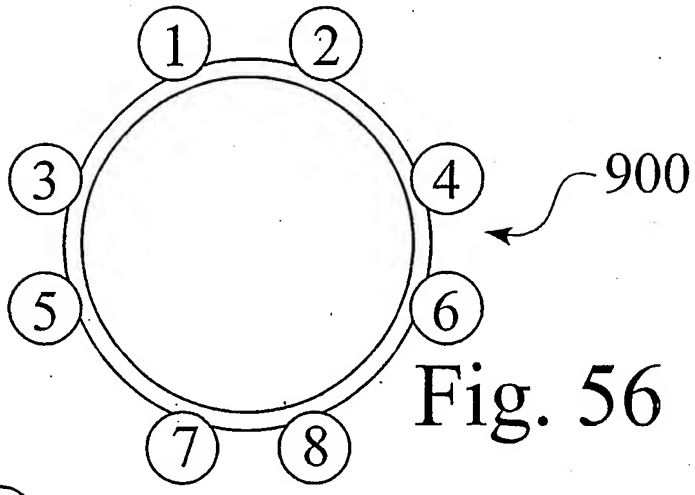


Fig. 56

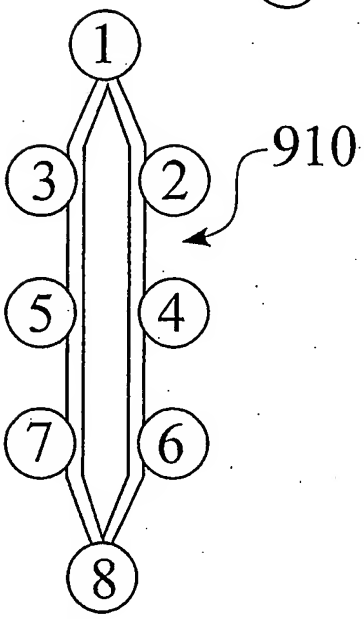


Fig. 57

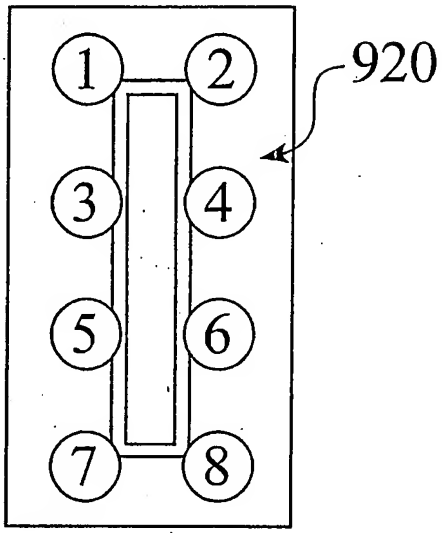


Fig. 58

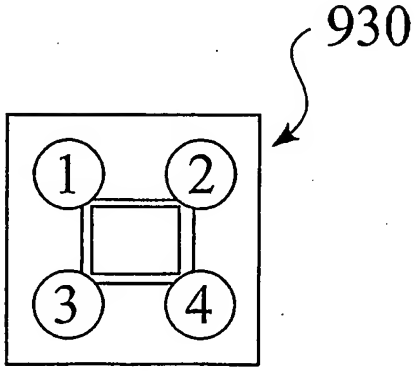


Fig. 59

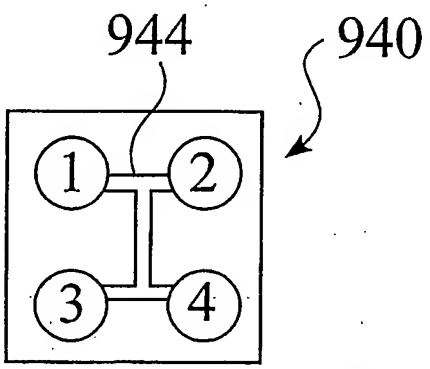


Fig. 60

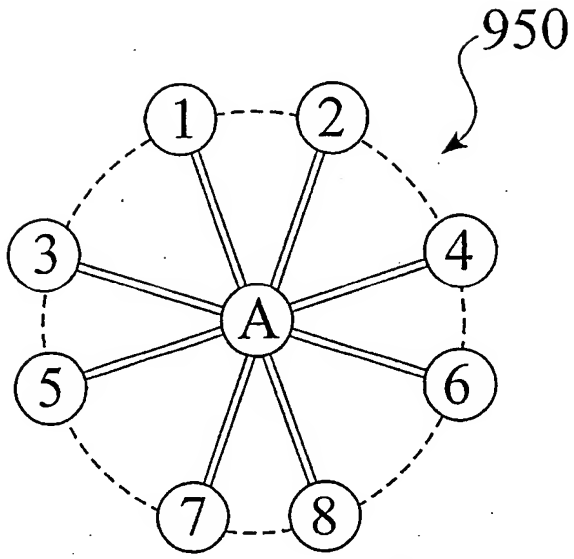


Fig. 61

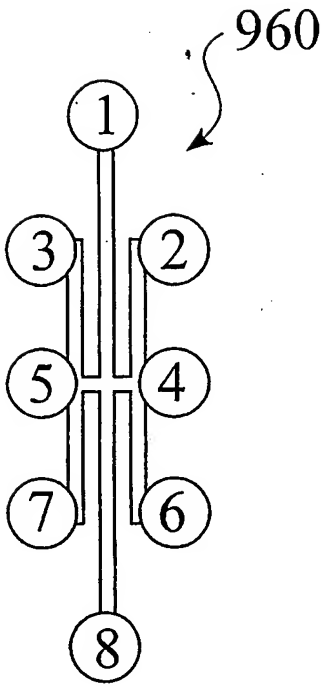


Fig. 62

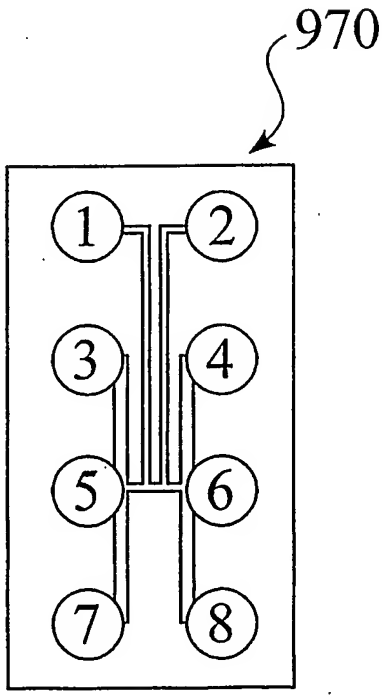


Fig. 63

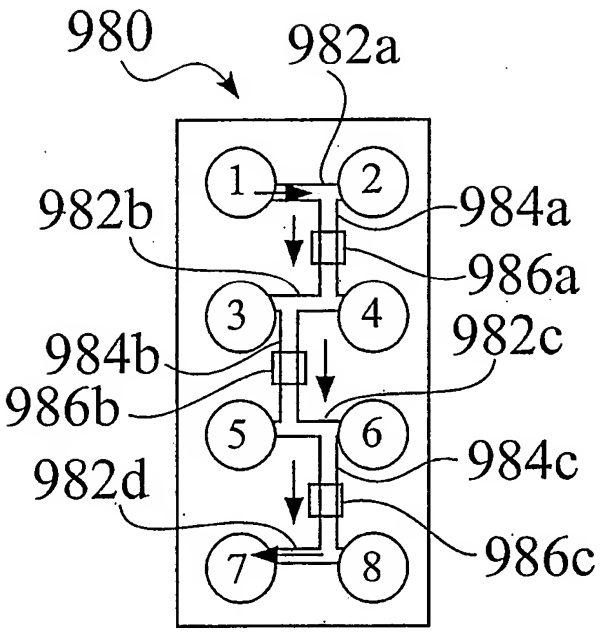


Fig. 64

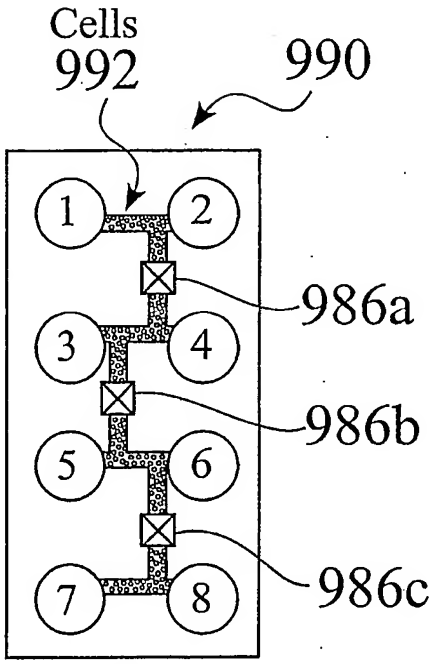


Fig. 65

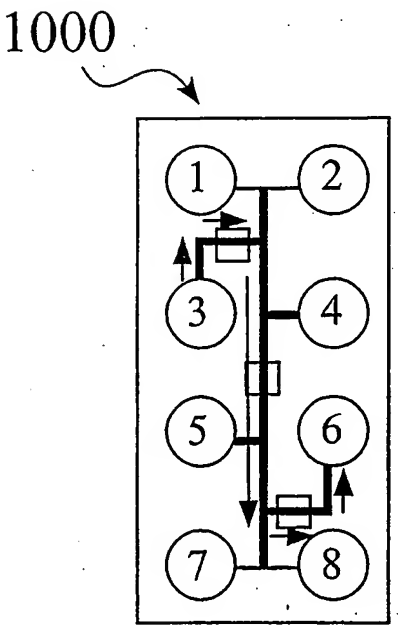


Fig. 66

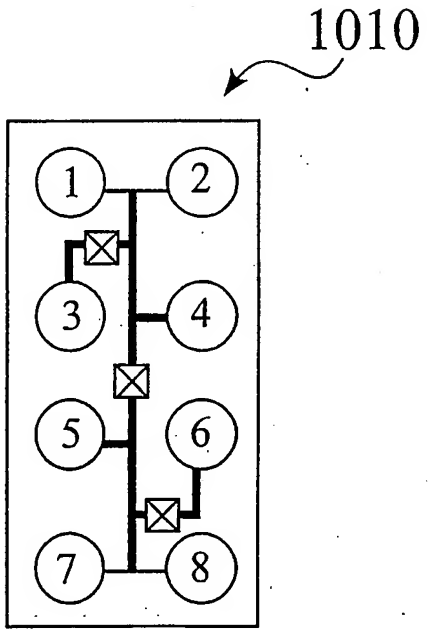


Fig. 67

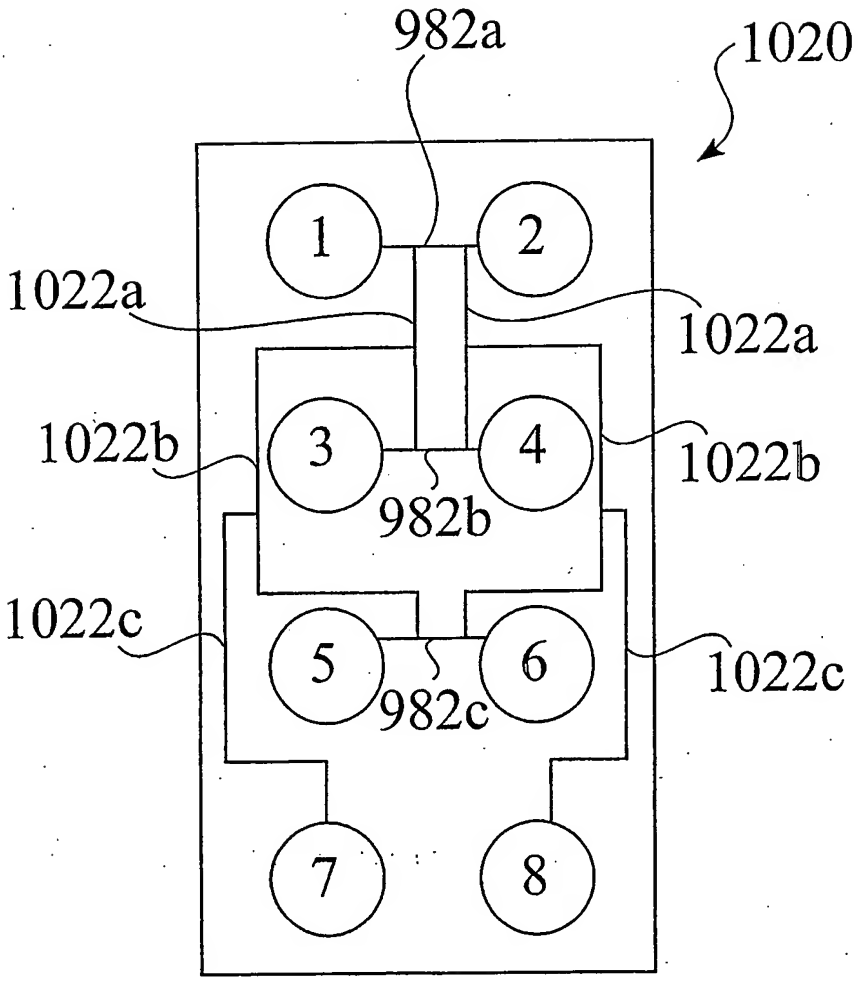

982a
1020
1
2
1022a
1022a
1022b
3
4
1022b
982b
1022c
5
6
1022c
982c
7
8

Fig. 68

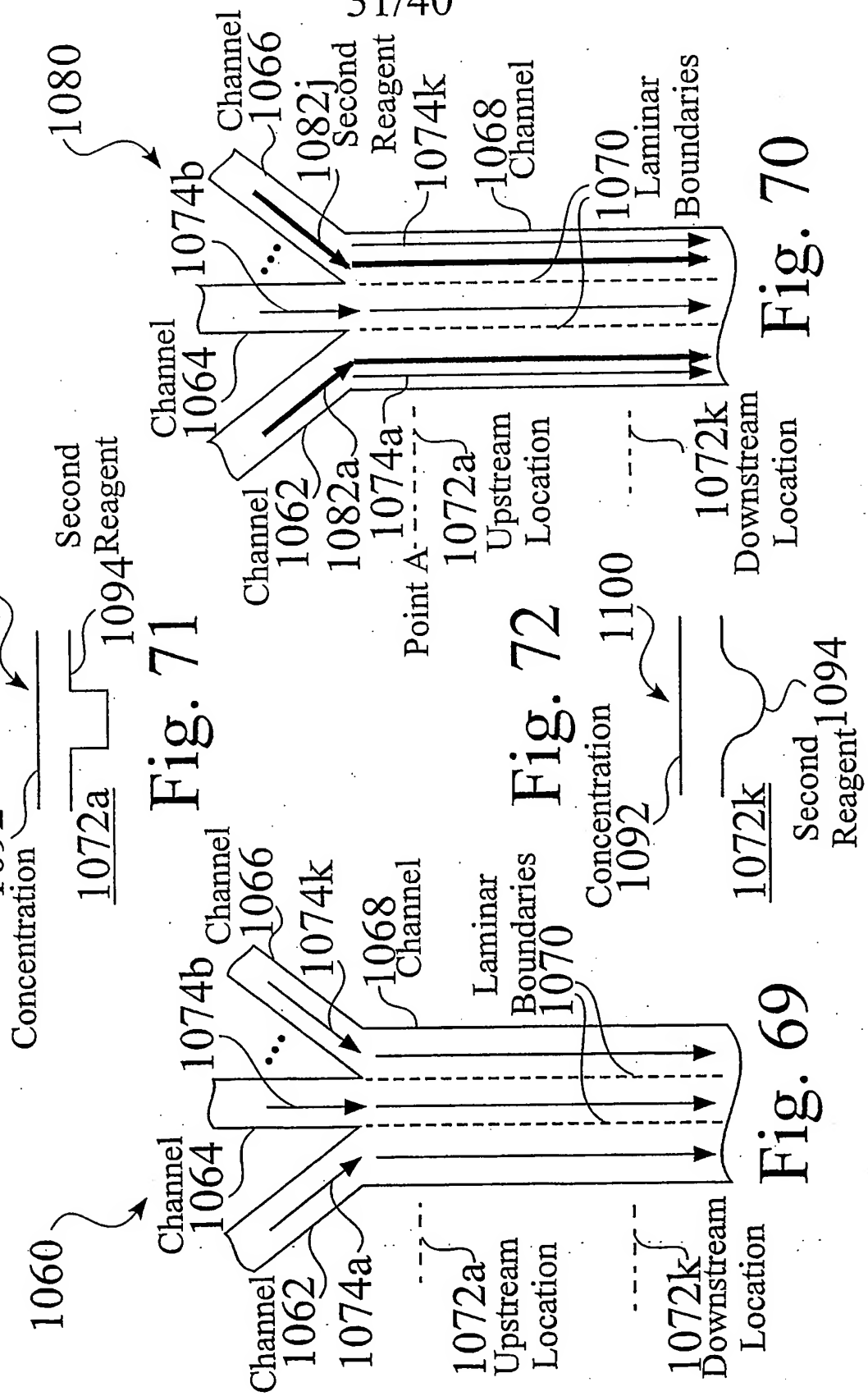


Fig. 69

Fig. 70

Fig. 71

Fig. 72

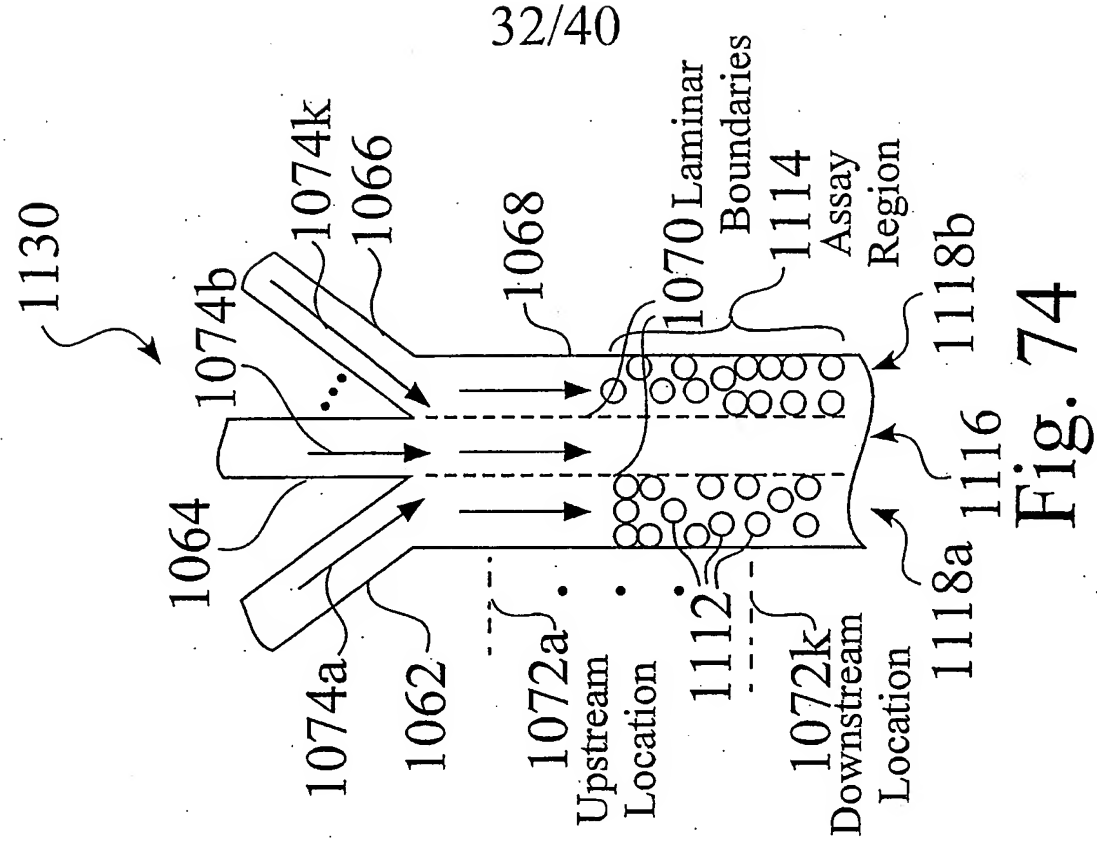

1130
1074k
1066
1074b
1068
1070 Laminar Boundaries
1114 Assay Region
1118b
1064
1116
1074a
1062
1118a
1072a Upstream Location
1112
1072k Downstream Location

Fig. 74

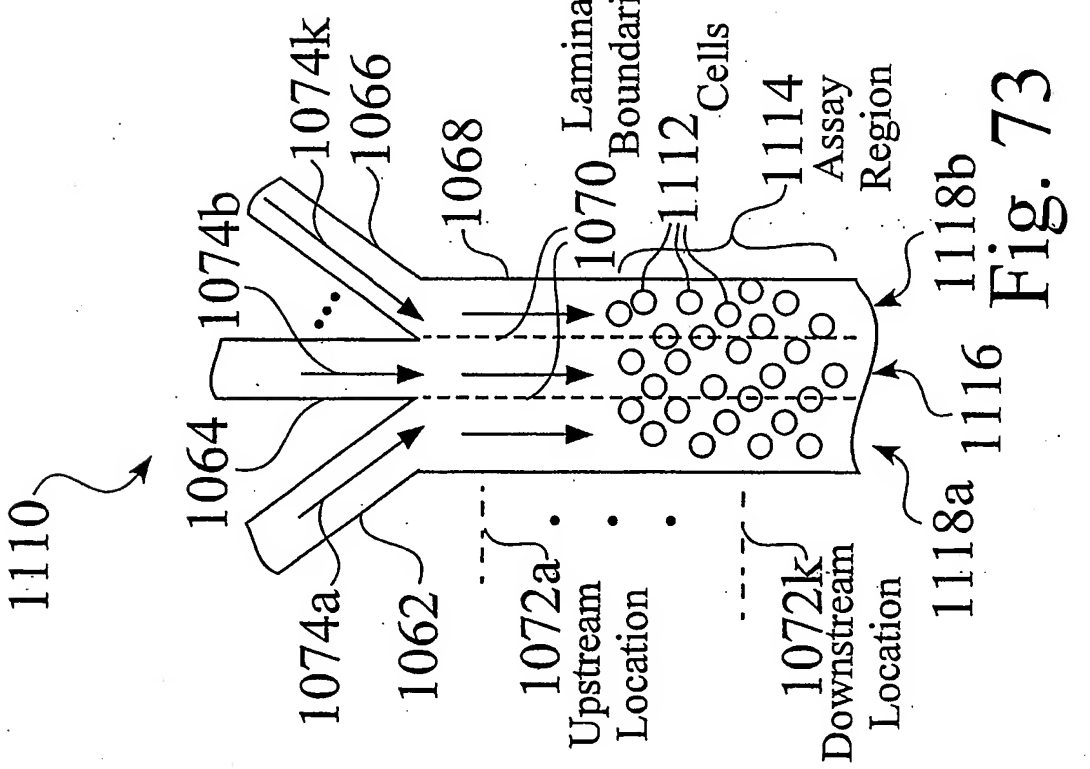

1110
1074k
1066
1074b
1068
1070 Laminar Boundaries
1112 Cells
1114 Assay Region
1118b
1064
1116
1074a
1062
1118a
1072a Upstream Location
1072k Downstream Location

Fig. 73

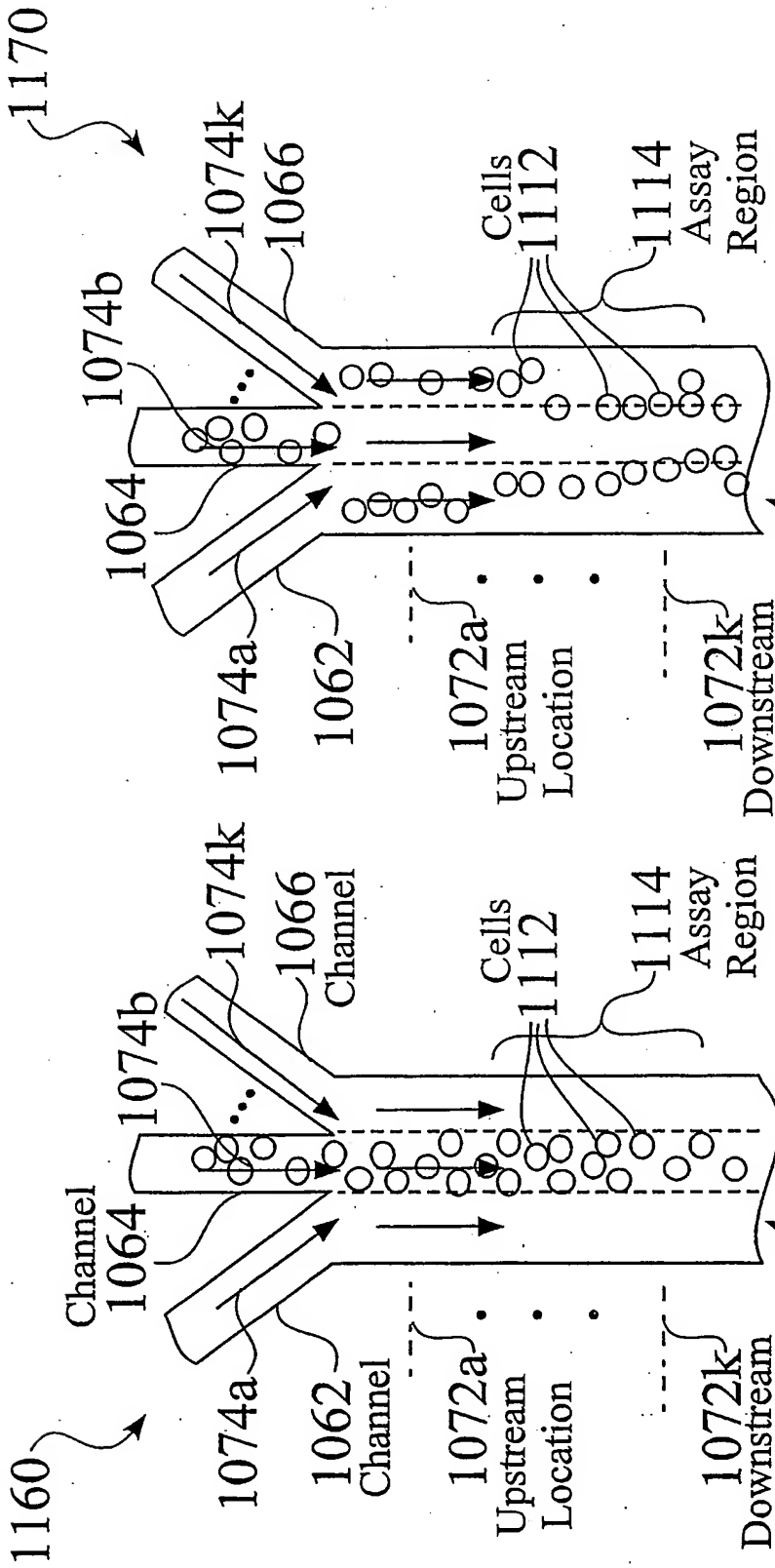
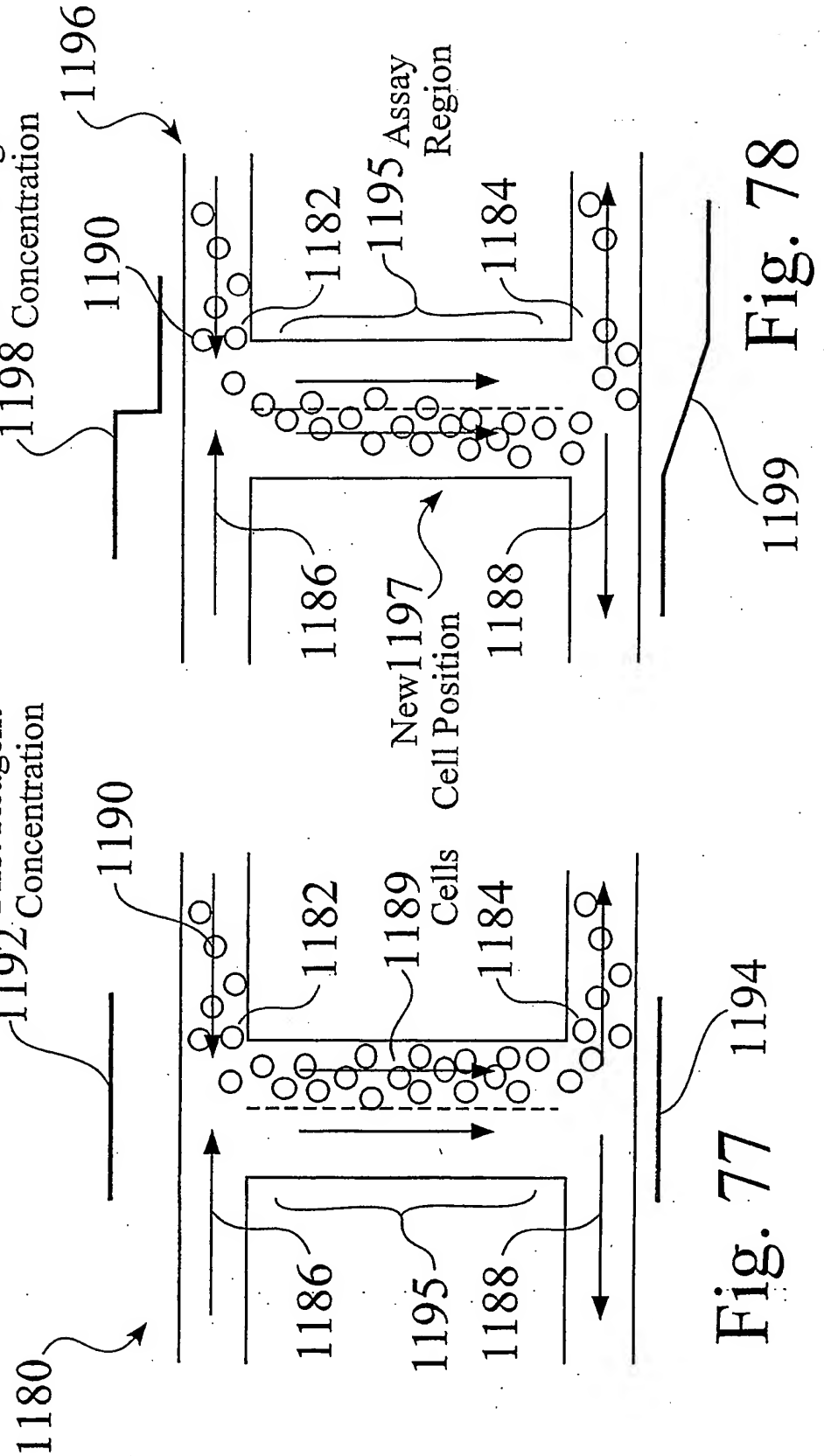

1160
1170
1074a
1062
Channel
1064
Channel
1074b
1066
Channel
1074k
1072a
Upstream
Location
1072k
Downstream
Location
Cells
1112
1114
Assay
Region
1116
1118a
1118b

Fig. 75

Fig. 76


1180
1192
First Reagent Concentration
1190
1182
1189
Cells
1184
1186
1195
1188
1194
Fig. 77
1196
Second Reagent Concentration
1198
1190
1182
1195
Assay Region
1184
1186
New Cell Position
1197
1188
1199
Fig. 78

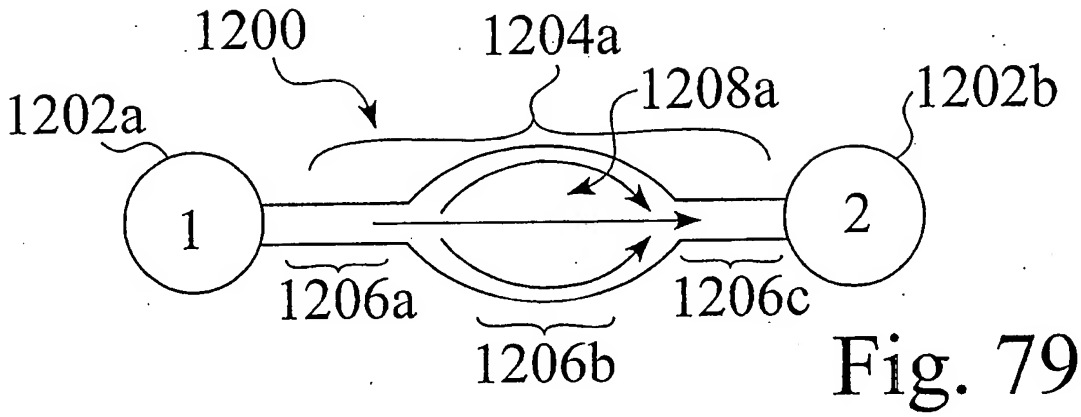


Fig. 79

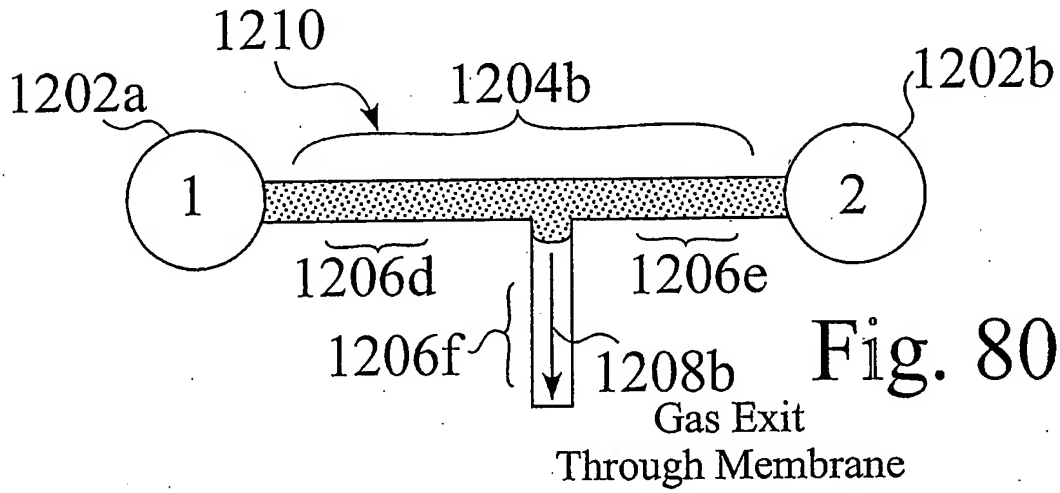


Fig. 80

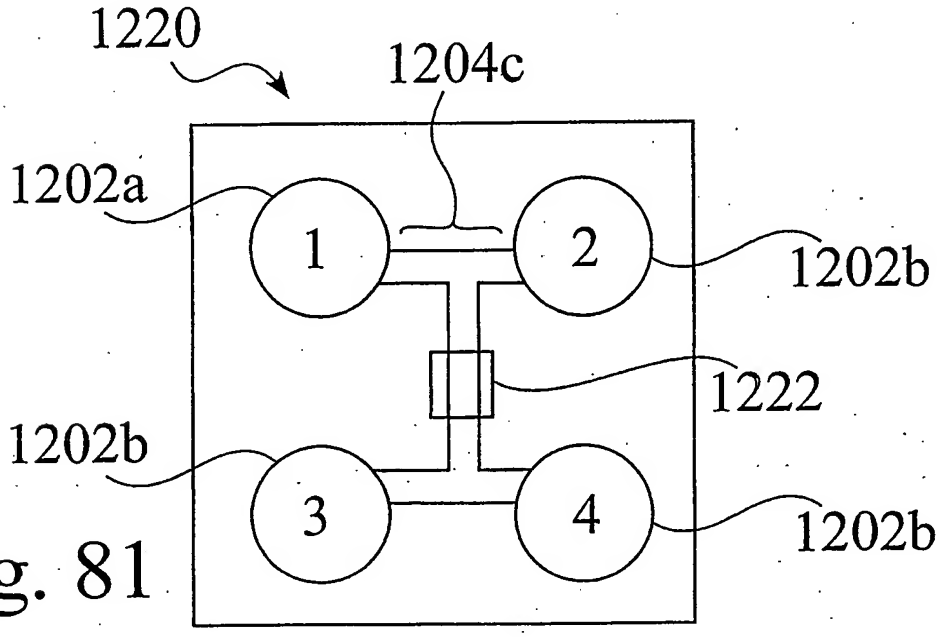


Fig. 81

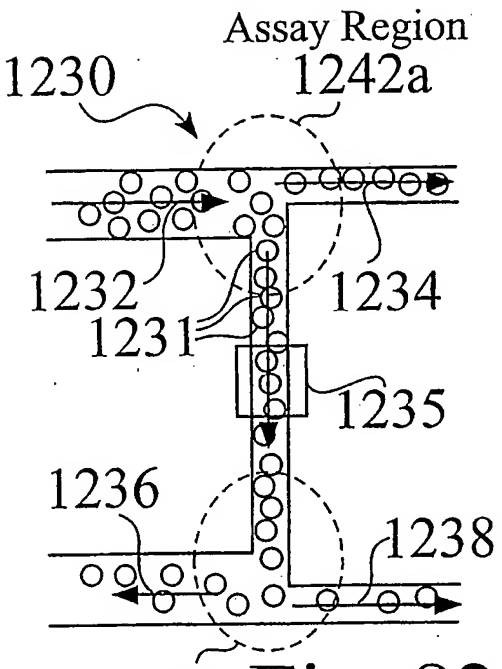


Fig. 82

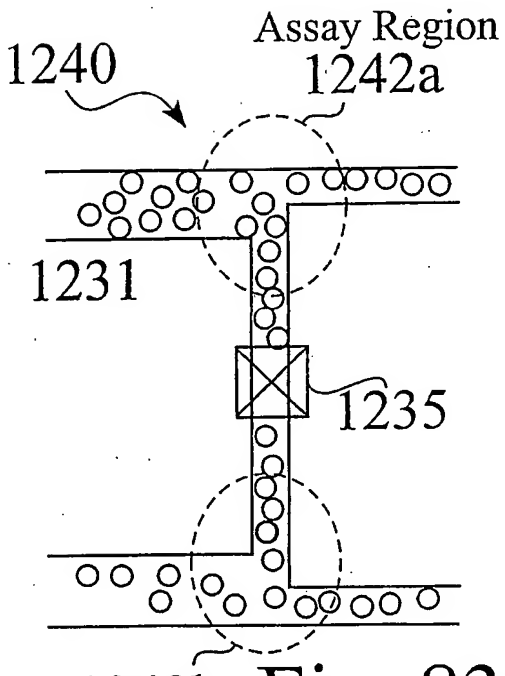


Fig. 83

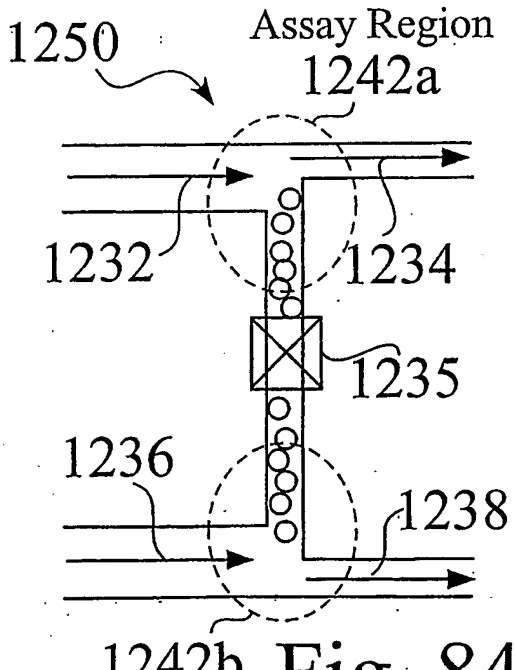


Fig. 84

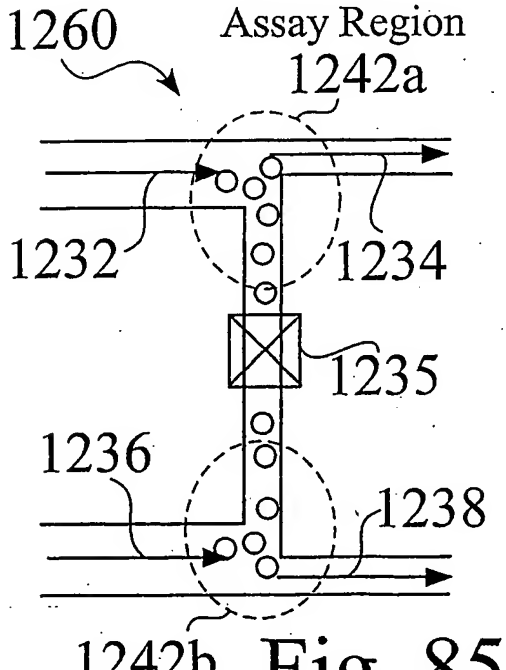


Fig. 85

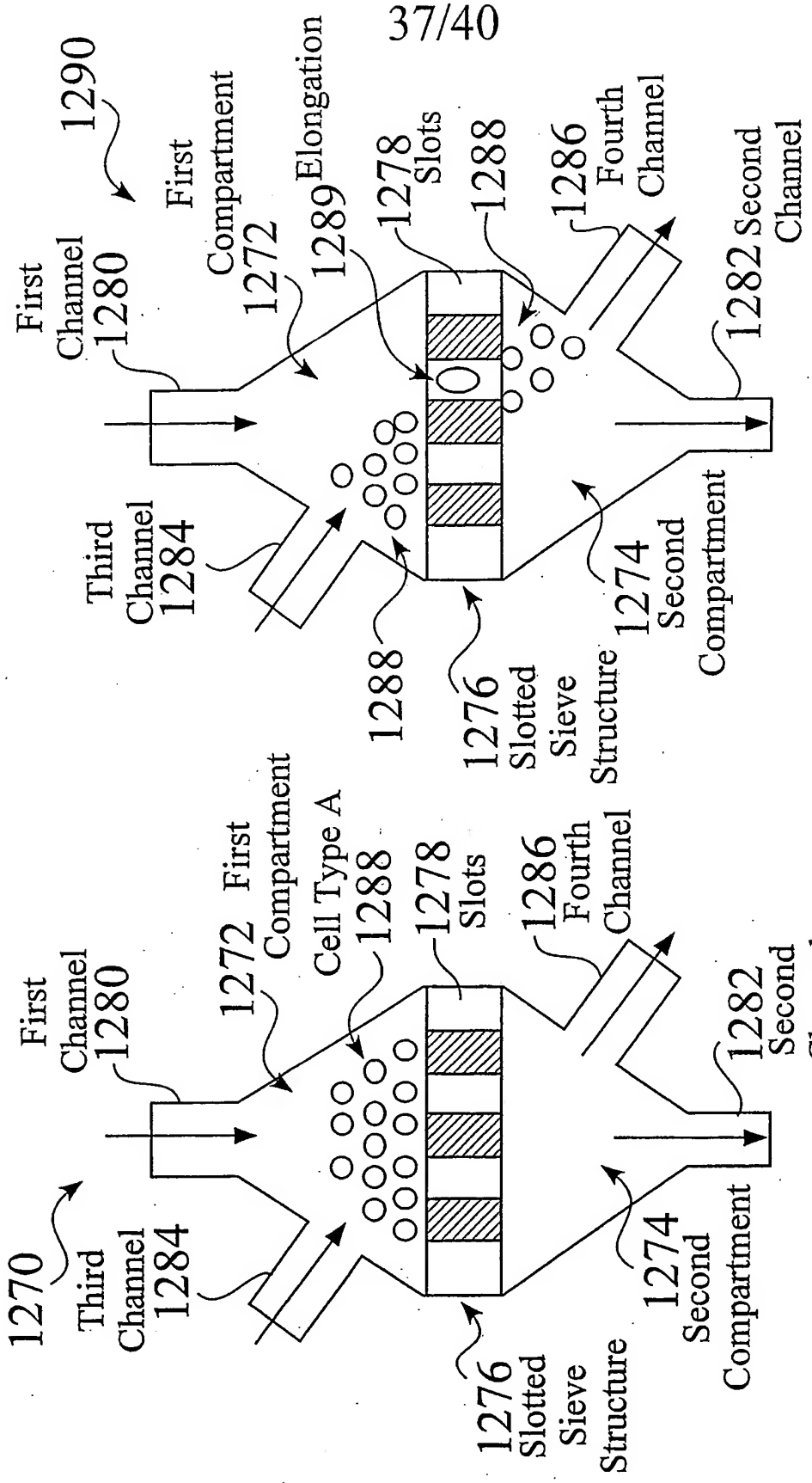

1290
First Compartment
Elongation
1289
1278 Slots
1288
1286 Fourth Channel
First Channel 1280
1272
1282 Second Channel
Third Channel 1284
1288
1276 Slotted Sieve Structure
1274 Second Compartment
1270
First Channel 1280
1272 First Compartment
Cell Type A
1288
1278 Slots
1286 Fourth Channel
1282 Second Channel
Third Channel 1284
1276 Slotted Sieve Structure
1274 Second Compartment

Fig. 87

Fig. 86

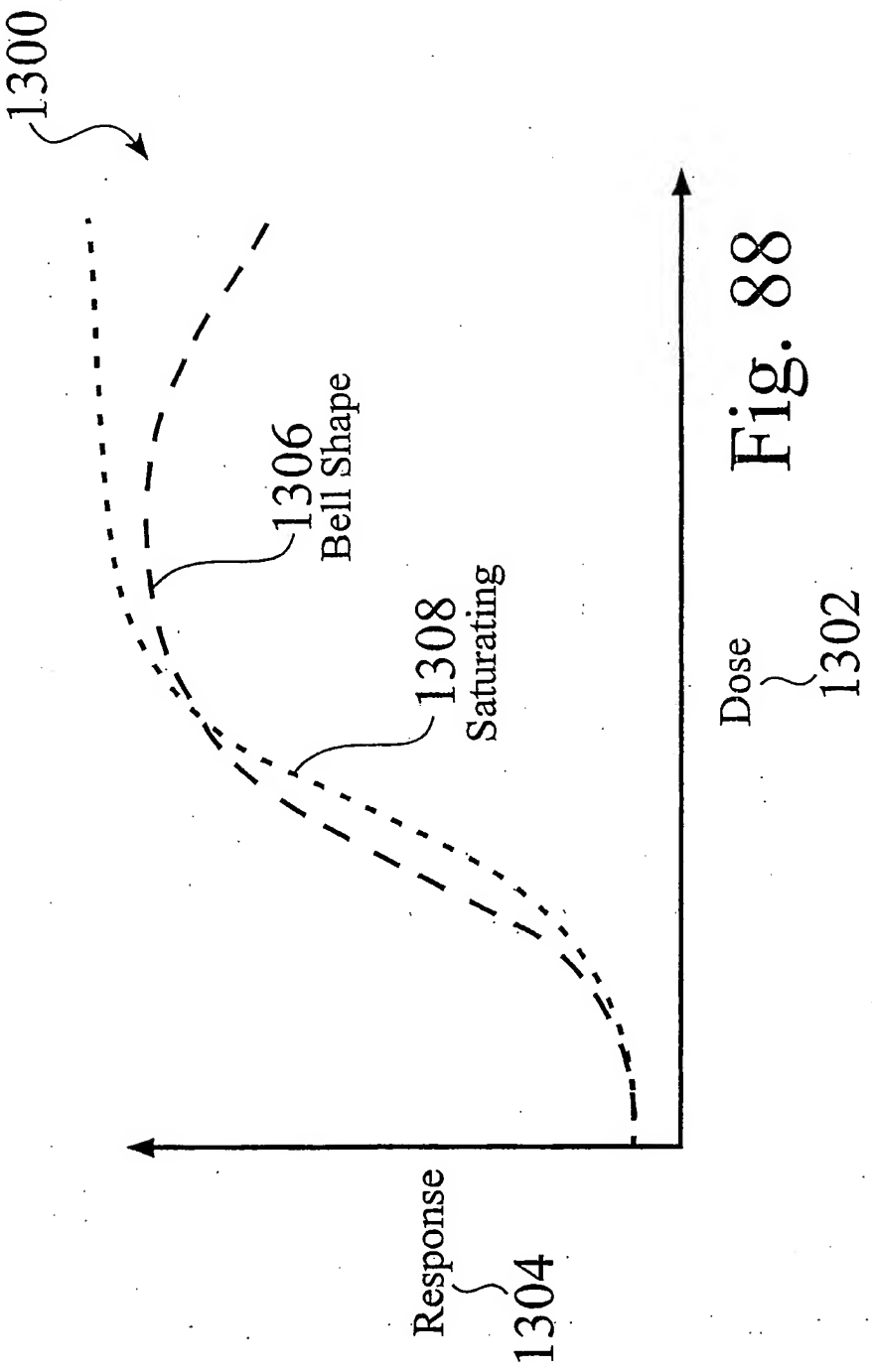

1300
1306
Bell Shape
1308
Saturating
Response
1304
Dose
1302

Fig. 88

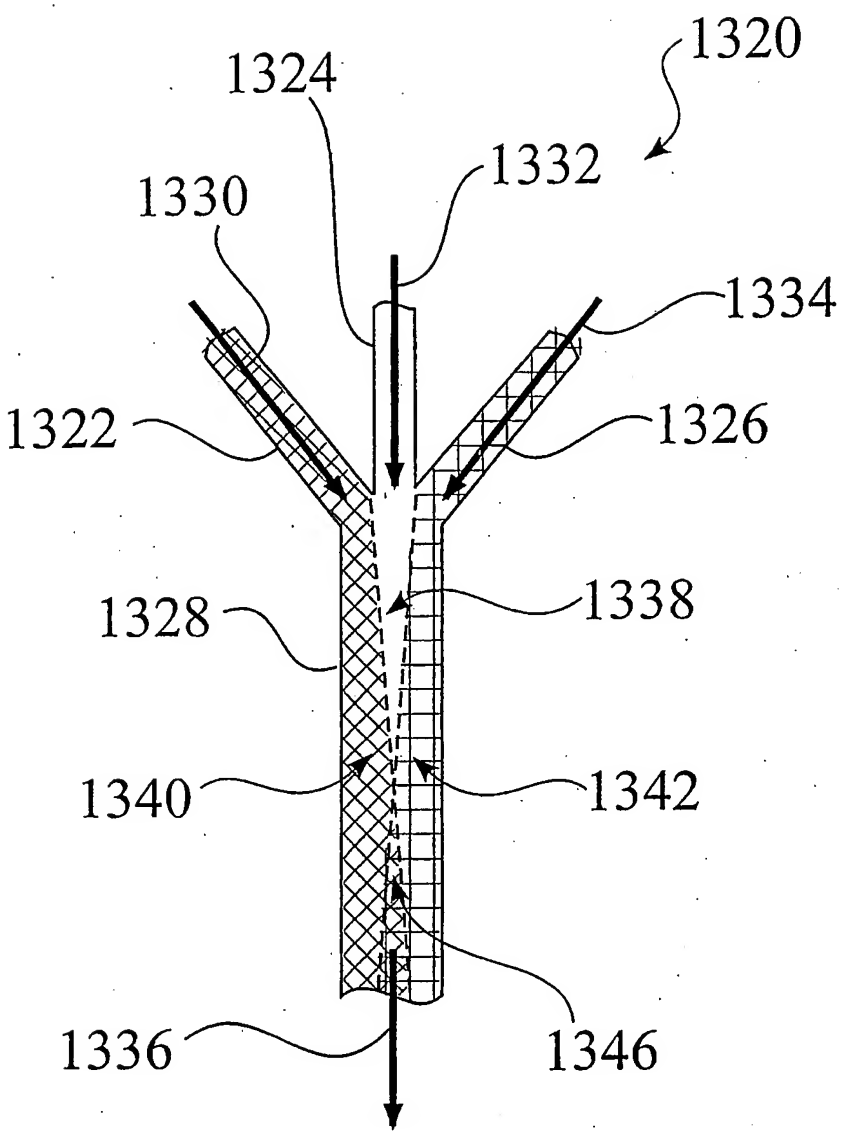

1320
1324
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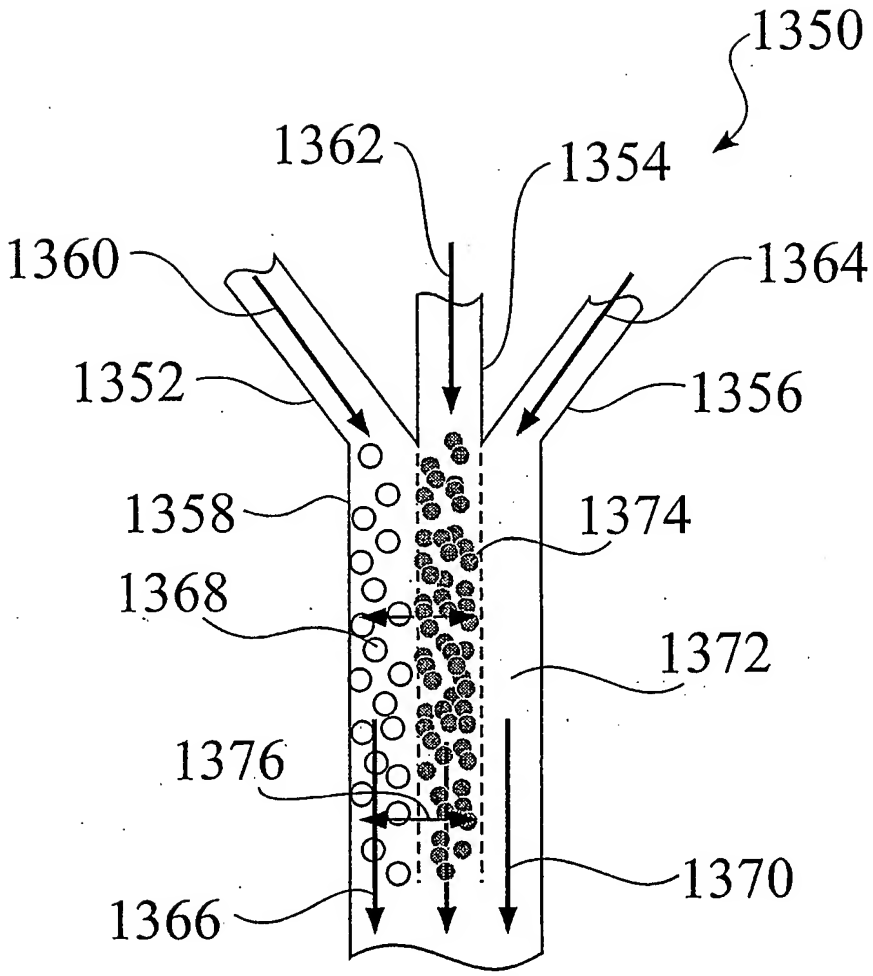
Fig. 89


1350
1362
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1356
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Fig. 90